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TOPIC HIGHLIGHT

Jaques Waisberg, PhD, *Series Editor*

Complications of radiofrequency ablation of hepatic tumors: Frequency and risk factors

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Abstract

Radiofrequency ablation (RFA) has become an important option in the therapy of primary and secondary hepatic tumors. Surgical resection is still the best treatment option, but only a few of these patients are candidates for surgery: multilobar disease, insufficient liver reserve that will lead to liver failure after resection, extra-hepatic disease, proximity to major bile ducts and vessels, and co-morbidities. RFA has a low mortality and morbidity rate and is considered to be safe. Thus, complications occur and vary widely in the literature. Complications are caused by thermal damage, direct needle injury, infection and the patient's co-morbidities. Tumor type, type of approach, number of lesions, tumor localization, underlying hepatic disease, the physician's experience, associated hepatic resection and lesion size have been described as factors significantly associated with complications. The physician in charge should promptly recognize high-risk patients more susceptible to complications, perform a close post procedure follow-up and manage them early and adequately if they occur. We aim to describe complications from RFA of hepatic tumors and their risk factors, as well as a few techniques to avoid them. This way, others can decrease their morbidity rates with better outcomes.

Key words: Radiofrequency ablation; Hepatic tumors; Complications; Risk factors; Hepatocellular carcinoma

Core tip: This article is an interesting and updated compilation of the complications of radiofrequency ablation of liver tumors. Several complications are described, as well as their risk factors and incidence. Some strategies to avoid them from happening are also reported.

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INTRODUCTION

Radiofrequency ablation (RFA) has become an important option in the therapy of primary and secondary hepatic tumors. Surgical resection is still the gold standard treatment, but only 5%-15% of these patients are candidates for surgery[1]. For a few selected patients who have hepatocellular carcinoma (HCC), the most common primary cancer, liver transplantation is an option but the inclusion criteria are strict and organ donation is still insufficient. Inadequate liver function, multilobar lesions, extra-hepat-

ic disease, proximity to major hepatic vessels and the biliary tract, and co-morbidities are factors that make these patients not eligible for surgery[2].

Complications rates of RFA vary widely in the literature. They are divided into major and minor[3]. The former are those that need some type of medical intervention (*e.g.*, drainage), increase morbidity and mortality, increase hospital stay or require blood transfusions. All of the rest are considered minor[3]. Authors have reported rates as low as 2% to 5.7% for major complications[4-6]. Mortality related to the procedure is low, reported in the literature to be less than 1%[7-9]. Tumor type, type of approach, number of lesions, tumor localization, underlying hepatic disease, the physician's experience, associated hepatic resection and lesion size have been described as factors significantly associated with complications[9-12]. In one of their papers, Poon *et al*[10] concluded that after the physician's first 50 procedures, the incidence of complications is lower, as well as a shorter hospital stay and higher complete ablation rate.

In this article, we present the frequency and risk factors for complications after RFA. Complications are summarized in Table 1.

Table 1 Complications of radiofrequency ablation

Hemorrhagic	Intra-abdominal bleeding
	Intra-hepatic bleeding
	Hemothorax
	Hemobilia
	Subcapsular hematoma
	Abdominal wall hematoma
Infection	Hepatic abscess
	Wound infection
	Sepsis
Biliary tract	Bile duct injuries
	Biliary stricture
	Bilomas
	Bilioperitoneum
	Biliopleural fistula
Liver failure	
Pulmonary	Pneumothorax
	Pleural effusion
	Pneumonia
Skin burn	
Tract seeding	
Vascular damage	Portal vein thrombosis
	Hepatic veins thrombosis
	Hepatic artery damage
	Pseudoaneurysm
Visceral damage	Colon
	Stomach
	Gallbladder
	Kidney
	Diaphragm
	Abdominal wall
	Small intestine

HEMORRHAGIC COMPLICATIONS

Intra-abdominal bleeding is the most common complication encountered in many studies[5,6,12,13]. In Mulier's review, it occurred in 0.7% of the procedures in 3670 patients[12]. Similar results were reported by Curley *et al*[9] (0.9% in 608 patients) and Livraghi *et al*[6] (0.5% in 2320 patients). It is believed to be a result of direct trauma from needle positioning rather than thermal injury (due to the protective "heat-sink" effect)[14,15]. Injuries to small vessels not visible on ultrasonography (US) are usually responsible for its origin. Increasing abdominal pain following the procedure is generally the most common symptom[9,15]. US or computed tomography (CT) confirms the diagnosis. Bleeding complications are more likely to happen in patients with HCC due to their underlying liver disease. In a study addressing this issue, tumor size, low platelet count and tumors located in segment VII were significant risk factors for intra-peritoneal bleeding[15]. Intra-hepatic bleeding may also occur and can be prevented by avoiding hepatic vessels while positioning the needle. This makes the imaging guidance essential. Both of them tend to have a benign course and stop spontaneously. Venous bleeding is usually treated conservatively or with blood transfusions only; arterial bleeding is more severe and may require surgical or endovascular intervention[9,14,16]. Tract cauterization by the withdrawal of the needle in high temperatures may prevent this kind of complication and should be performed in all cases[12]. Groups performing this have less or even no bleeding complications[7]. Rhim, in one of his articles, states that the open or the laparoscopic approach can decrease this kind of complication since needle positioning and withdrawal is under direct vision[16]. Transcatheter arterial embolization is the treatment of choice for this hemorrhagic complication[9,14,17].

Several authors have also described hemothorax[8,12,15]. It is less frequent than intra-abdominal bleeding, with an incidence ranging from 0.1% to 0.3%[8,12,15,17]. It usually occurs due to injuries to intercostal arteries while percutaneously ablating tumors in the right liver through an intercostal approach. Chest pain and dyspnea are the most common symptoms[15]. US, chest CT and chest X-Ray confirm the diagnosis. Circulation stabilization and thoracic drainage are often necessary[15]. An open approach for these patients should prevent this from happening.

Another hemorrhagic complication is hemobilia, with an incidence from 0.1% to 0.5%[12,15]. It is caused by the puncture at the same time of the biliary tract and a vessel[15]. The most common symptoms are abdominal pain, hematemesis and melena. The main risk in these cases is biliary obstruction by blood clots, causing jaundice and liver failure. In this matter, the timing of drainage is essential. Goto *et al*[15] indicates bile duct drainage when bilirubin concentrations exceeds 4 mg/dL; they think that an early indication of the procedure may delay hemostasis. They also found that tumors in liver segment I was a significant risk factor for this type of bleeding. Avoiding puncturing dilated biliary radicles should prevent such complications to occur[14].

Subcapsular hematoma and abdominal wall hematoma have also been described. The first one occurs more often in subcapsular tumors, when tract cauterization is

not possible, due to its depth. The open or laparoscopic approach rather than the percutaneous is an option to avoid them.

This illustrates the need for vigilance for any signs of bleeding after the procedure and adequate screening for coagulation disorders, including the use of medications that affect the coagulation cascade[18,19]. Post procedure imaging is also essential since these complications usually occur in the first hours after the ablation.

INFECTION

Abdominal infection is also a common complication encountered[12,20]. This group of complications consists of hepatic abscess, wound infection and sepsis. Hepatic abscess is a potentially dangerous complication with an incidence ranging in the literature from 0.3% to 1.7%[6,9,11,12,21-23]. It can appear up to more than 60 d after the procedure[23]. Significant risk factors for its development are the presence of biliary abnormality or manipulation, prone to ascending biliary infection (bilioenteric anastomosis, endoscopic papillotomy and tumor with retention of iodized oil from a previous chemoembolization)[6,12,16,22,24]. In a study conducted by Elias *et al*[23] in 2006, the authors studied 11 patients with enterobiliary anastomosis or biliary stent and found an incidence of 44% of hepatic abscess in these specific subjects. They also stated an interesting issue: when the biliary procedure was synchronous with the RFA, no hepatic abscesses were observed; only when it was performed prior to the ablation was it considered a risk factor. Enteric bacteria coming from the injured colonized bile ducts contaminate the tumor necrosis generated by RFA[22]. Patients with hepatic abscess may present with fever and abdominal pain. The onset of these symptoms and signs usually occur within the first month after RFA[22]. Suspicion should arise when patients present with high body temperatures after the procedure, especially if it lasts longer than two weeks, although fever can be a symptom of the postablation syndrome. CT scan confirms the diagnosis; air bubbles are usually seen in the abscess. Thus, they may be seen in the ablated area after the procedure and this must not be misdiagnosed as an abscess[20]. Antibiotic prophylaxis is controversial in all patients, but in high risk cases it is recommended[6,12,22,23]. A question that comes up in these patients is if prolonged antibiotic prophylaxis is useful in reducing its incidence. Hoffmann *et al*[24] addressed this issue and tried to reduced this risk by maintaining the antibiotics for over 10 d after the procedure in 8 patients with prior bilioenteric anastomosis. The majority of the interventions (9/10) had prior administration of intravenous piperacillin/tazobactam and after the RFA, patients received Ciprofloxacin orally; 4 of the patients received additional antibiotics (metronidazole, cefpodoxime and cefazolin). Only one patient developed a hepatic abscess; he had a chemoembolization 8 d before the RFA. Despite the low number of patients and the lack of a control group, the authors suggest that this regimen may decrease the incidence of hepatic abscess. Elias *et al*[23] and de Baère *et al*[5] also debated this matter. Both groups administered prolonged antibiotics prophylaxis for 5 d (longer than usual) on these high-risk patients and a high incidence of hepatic abscess was encountered. Further studies with control groups and larger series of patients are necessary to resolve this question.

The most frequent organisms found in these abscesses were *Enterococcus*, *E. coli, Bacteroides fragilis*, *E. faecalis*, *C. perfringens* and *Klebsiella pneumonia*[5,21,23]. The best treatment option is percutaneous drainage in combination with systemic antibiotics[19-21,24]. Early suspicion, diagnosis and treatment are essential for a good outcome so the physician should be alert to the patient's clinical follow up, especially in those with risk factors.

BILIARY TRACT DAMAGE

Biliary tract damage includes bile duct injuries, biliary stricture, bilomas and, most rarely, bilioperitoneum and biliopleural fistula. Its incidence can be as low as 0.1% and up to 12%[9,12,25,26]. Bile ducts changes are expected and most of these changes have no clinical significance with the patient being asymptomatic with low rates of progression[9,12,26]. This explains its low and underestimated frequency since authors ignore those minor changes[12,26]. In a paper studying this matter, most of these changes seen on CT were mild dilatation of the upstream intrahepatic bile duct surrounding the ablation zone[26]. The authors did not mention the distance between the tumors and major bile ducts and stated that these changes are irreversible. In an Italian study, only two of 3554 patients required therapy after this kind of complication[6]. Another 15 patients presented with asymptomatic biliary tree abnormalities. These injuries are due to thermal damage from heating and direct mechanical damage from the needle. It is more likely to happen in hilar tumors or in tumors closer than 1 cm to major bile ducts when the safety margin is impossible to be obtained without injury. Biliary stricture is the most common complication in this group[12]. It may develop weeks to several months after RFA[26]. In a study where 28 high-risk patients were analyzed, the incidence of stenosis in this specific group of subjects increased up to 46% (13/28 patients with tumors closer than 5 mm to central bile duct on CT)[25]. Peripheral stenosis is usually asymptomatic, but central strictures may lead to serious complications. These strictures are believed to lead to liver atrophy and its consequent malfunction[25]. This is very important for cirrhotic patients because, due to their already impaired liver function, they may easily develop liver failure and cholangitis after bile duct stenosis[21,25]. Cholestasis and biliary infection may also occur.

Diagnosis is usually done by CT during follow-up and can also be detected by endoscopic retrograde cholangiography. The latter can also be used therapeutically by stenting the injured bile duct. The strictures are also well treated by endoscopic sphincterotomy[27].

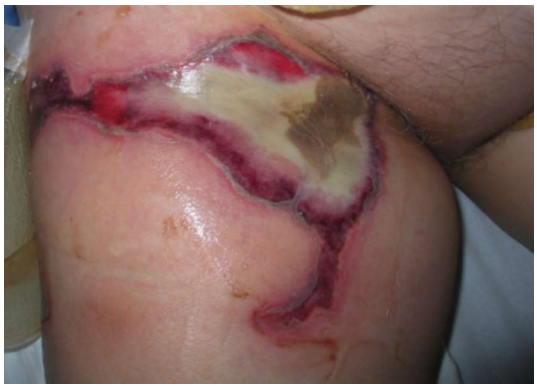

Figure 1 Third-degree grounding pad skin burn on the right thigh.

The association of RFA with transarterial chemoembolization (TACE) or percutaneous ethanol injection (PEI) is an option in these cases as these procedures, prior to the RFA, decrease tumor size and makes it possible for the ablation to be safer with a larger margin. Ohnishi *et al*[25] reported a method to prevent this complication by infusing intraductal chilled saline solution through an endoscopic nasobiliary drainage tube. Only one patient (2.5%) developed a stricture (left hepatic duct); the 39 remaining subjects were able to avoid thermal injury with this procedure. The incidence of this complication was significantly lower than the control group. This also significantly decreased the worsening of their liver function compared to the control group. The authors did not mention recurrence and other complications related to this procedure. Elias *et al*[23] also used this in 13 high-risk patients after the procedure. Two questions arise. The first one is if this protection is due to the low temperature itself or the heat sink effect caused by the solution's flow leading to inefficient ablation. The second one is if this procedure increases the incidence of hepatic abscesses. These questions need to be answered with future studies. Another concern regarding this issue is recurrence. This procedure also has a cooling effect on tumor cells near the cooled bile duct; thus, more insertions and more heat are necessary for adequate ablation which may lead to higher rates of complications[28]. Future studies are needed to address this. Curley *et al*[9] and Huang *et al*[29] suggested an open approach in these high-risk subjects for better needle placement with intra-operative ultrasonography. Patient selection is vital to avoid this type of complication.

Biloma is also encountered in this group of complications, with an incidence ranging from 0.1% to 5.8%[6,12,26,30]. It is defined as an encapsulated bile collection outside the biliary tree due to biliary leakage. This leakage can be caused by direct damage from the needle, direct thermal damage and by thermal damage to the microvasculature of the biliary tract caused by RFA. On CT, it is characterized as a circumferential fluid collection surrounding the ablation site or a communication between the bile duct and circumferential collection confirmed on cholangiography or CT[26,30]. Most bilomas develop within the first 4 mo but can occur as late as 17 mo[30]. Almost all patients are asymptomatic and the fluid formation has spontaneous regression in half of the cases[30]. Percutaneous drainage is a good treatment option when required. Sphincterotomy should always be considered to exclude biliary stenosis and increased biliary pressure as a cause for biloma formation.

LIVER FAILURE

Liver failure is also a potentially fatal complication, especially in patients with cirrhosis whose liver function is often already impaired. Patients who have undergone previous hepatectomy are also at risk for this complication[14]. Its incidence ranges from 0.2% to 4.3%[4,9,11,12]. Child Pugh classification has been significantly related to post treatment liver failure[4,11]. Hepatic infarction due to injuries to major feeding vessels is believed to be responsible for its occurrence. Proper and careful needle placement is essential to avoid this from happening[14]. Other causes of liver failure are extensive ablation (overtreatment causes destruction of cirrhotic tissue around the lesions), portal vein thrombosis and extensive resection[6,12,16].

PULMONARY COMPLICATIONS

Pneumothorax, hemothorax (described in hemorrhagic complications), pleural effusions and pneumonias are in this group of complications. Its incidence varies from 0.8% to 2.1%[9,12]. Pneumothorax is more likely to happen in patients with tumors located directly under the diaphragm when an intercostal approach is chosen[12]. Some authors have described the use of artificial pleural effusion[28]. The idea is to separate the lung from the diaphragm and avoid these lesions. Inoue *et al*[28] published a series of 64 patients with 82 nodules near the diaphragm using this technique and encountered complications in 5 subjects. The treatment should be considered individually. Thoracentesis, underwater seal drainage and diuresis have been described[6,9,14]. Adequate needle positioning with a safe window (in the percutaneous approach) can avoid this complication[20]. Positioning the patient on the right side can also avoid it by limiting respiratory excursion[14]. Use of the epipericardial fat pad has also been described to avoid entering the pleural cavity[31]. Further investigation with CT is required if the patient experiences dyspnea or chest pain after RFA.

SKIN BURNS

Skin burns can occur at the point of needle entry and at the ground pad sites (Figure 1). This complication had a higher incidence in earlier studies due to smaller pads. In recent papers, it became a rarity because of their larger sizes and increased awareness, with a low incidence from 0.2% to 0.6%[6,14,20]. Third-degree skin burns are rare, but have been described, even leading to deaths[5,7,19,20]. Adequate pad placement and sizes are essential to avoid

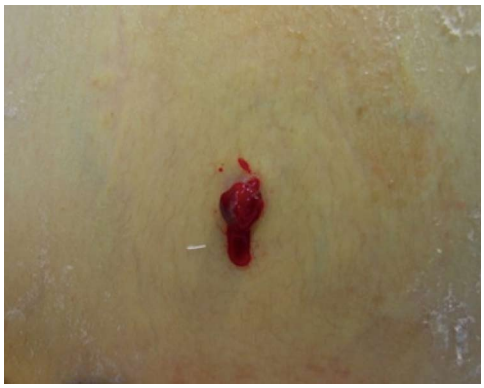

Figure 2 Tumor seeding on needle entry site after percutaneous radiofrequency ablation.

this complication, as well as good contact with the skin. Large and sometimes multiple ground pads are necessary to disperse the high amount of energy generated by RFA. They should be equidistant from the needle due to the asymmetric distribution of the electrical current. This asymmetry makes the temperature beneath the pads not uniform, with greater heat on the edges and in the pads closest to the needle[19]. This was confirmed by de Baère *et al*[5], describing patients with first and third degree burns on the edges of one the pads facing the active electrode (needle).

TRACT SEEDING

Tumor seeding in the needle tract has an incidence from 0.2% to 0.9%[5,6,12,32]. Low rates of tumor seeding may be explained due to its underestimation in most papers due to a lack of follow-up. It usually occurs 3 to 12 mo after RFA[19]. Viable tumor cells that adhere to a biopsy needle or the electrode during its extraction, tumor cells carried into the needle tract with the bleeding and tumor cells forced into the tract by intratumoral hyperpressure are mechanisms that explain the seeding[12,33] (Figure 2). Decreasing the number of punctures and transversing a large amount of hepatic tissue before entering the tumor may avoid this complication[14,20]. Groups performing needle tract cauterization have not experienced tumor seeding or have very low rates[5,7,14]. Livraghi *et al*[32] reported their series with 1314 patients aiming to determine the risks of this complication in subjects with HCC treated by percutaneous RFA with a long follow-up (median 37 mo). They encountered seeding in 12 patients; tumors were located mostly in intercostal muscle and successfully treated by resection. The only significant risk factor described was a previous biopsy. They concluded that needle biopsy should be avoided. Other risks factors described by other authors are poorly differentiated, subcapsular location (where heating of the needle tract is not possible) and multiple needle insertions[5,6,33,34]. Optimal and meticulous first attempt electrode positioning is desirable[6]. Besides resection, RFA is also an option for treating tumor seeding. Some authors suggest the open approach in subcapsular lesions to avoid this complication[35].

HEPATIC VASCULAR DAMAGE

Portal vein thrombosis, hepatic vein thrombosis, hepatic artery damage and pseudoaneurysm represent this group of complications, with an overall complication rate from 0.5% to 1%[6,12,19,36].

Portal vein thrombosis is a potentially fatal complication, with a 0.2% incidence[12]. Thrombosis and coagulation of vessels larger than 3 mm are rare when normal flow is granted[37]. Most of these thromboses are asymptomatic even in larger vessels and no further therapy is required[5,36]. They are caused by heat damage to the endothelial cells of the portal or hepatic vein, leading to platelet aggregation and subsequent thrombosis[38]. It can be defined as being adjacent to the ablation zone and developing within 4 mo after RFA[36]. Liver function tests are usually normal but if elevated should normalize with no clinical significance[36]. Its occurrence should be avoided, especially in cirrhotic patients, as it may lead to liver failure in a patient with an already impaired liver function. Risk factors are the central location of the tumor, vein compression by the tumor and the Pringle maneuver. The latter stops blood flow into the liver and with that, vessels lose their cooling protection from the "heat-sink" effect, leading to vessels thrombosis. de Baère *et al*[5] showed in their paper that 30% of their procedures with balloon occlusion (for blood flow stop) led to complete thrombosis of the ballooned vessel. They also had more significant portal vein thrombosis in cirrhotic patients after performing the Pringle maneuver than in noncirrhotic subjects. It is suggested by the authors that it should be avoided in these patients, even for short durations[5].

Hepatic artery damage has a 0.2% incidence[12]. Small arterioportal shunts may occur after RFA and the majority of them heal spontaneously[12]. They can be successfully treated by endovascular or percutaneous therapies.

VISCERAL DAMAGE

Visceral damage is rare, with an incidence varying from 0.5% to 0.7%[6,12]. Damage to the colon, stomach, gallbladder, kidney, diaphragm, abdominal wall and small intestine has been described. Attention should be paid when tumors are closer than 1 cm to adjacent organs. Early diagnosis and adequate treatment are essential since it may lead to death. Risk factors are percutaneous approach, subcapsular tumors, previous abdominal surgery and chronic cholecystitis as the patient may have adhesions between the liver and the bowel[6,12,16]. Livraghi *et al*[6] suggest some issues in these patients: they should be treated by the open or laparoscopic approach for direct visualization of the organs, assuring they are in fact separated, and CT guidance is preferable for better adjacent bowel identification.

The colon is believed to be at greater risk of being

damaged due to its thin wall and fixed nature[5,6]. This complication has an incidence from 0.1% to 0.3%[5,6,12]. Some techniques have been developed to avoid bowel injuries: patient positioning in a steep oblique and prone position and breath holding during mechanical ventilation in patients under general anesthesia has also been described[14]. Another technique is creating a barrier between the liver and the colon, the hydrodissection. The use of 5% dextrose and saline solutions has been reported[14,28,39]. The former is preferred due to its properties since it does not conduct electricity and hence provides a thermal barrier around the organ[39]. Song *et al*[39] and Inoue *et al*[28] used artificial ascites and had no gastrointestinal injuries. The stomach and small bowel are less injured because adhesions along the gastrohepatic ligament are rare as the gastric wall is very thick and the small bowel has great mobility and peristalsis[6,19]. One should keep in mind that the onset of the symptoms of perforation is delayed; therefore, treatment is also usually delayed and the patient presents with a severe clinical status, eventually leading to death. A high level of suspicion is essential and close follow-up is important in these subjects.

Ribeiro *et al*[7] in their series routinely performed open cholecystectomy prior to RFA in tumors near the gallbladder, with the intention to avoid cholecystitis and incomplete ablation. Minimal wall thickening is expected on imaging after RFA, usually with no clinical significance. This probably happens due to the capacity of the fluid inside the gallbladder to dissipate the heat[16].

Injury to the diaphragm occurs in 0.1% of the cases[6,12]. It frequently results in severe shoulder pain[14]. Usually, RFA causes thickening of the muscle but perforation and hernia have been described[40]. Artificial ascites can also be used to decrease it.

CONCLUSION

Complication rates of RFA are low, making it a safe and feasible procedure. Every component of the treatment should be thoroughly analyzed. Proper patient selection is essential; subjects with exclusion criteria may lead to higher complication rates. Type of approach is also vital; depending on tumor location, one type may lead to a higher complication rate than another. This also fits for imaging guidance, where some tumors locations are better visualized by a specific method over another. The physician's experience is very important as well. Identification of high-risk subjects (with close follow-up), early diagnosis of known complications and a high level of suspicion are acquired with time and may lead to better outcomes and reduced risk of complications.

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REVIEW

Thyroid hormone analogues and derivatives: Actions in fatty liver

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Author contributions: Coppola M and Glinni D contributed equally to this work; Coppola M wrote the paragraph: Naturally occurring iodothyronines and liver steatosis: 3,5-T2; Glinni D wrote the paragraph: Actions of T3 on lipid metabolism in the liver: implications for fatty liver; Moreno M wrote the paragraphs: Introduction and Conclusions; Cioffi F wrote the paragraph: Thyroid hormone functional analogues: TRC150094 (TRC); Silvestri E wrote the paragraph: Thyromimetics and liver steatosis and realized the figures; Goglia F coordinated all the work and revised all the manuscript.

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Abstract

Fatty liver or nonalcoholic fatty liver disease (NAFLD), a problem of increasing clinical significance and prevalence worldwide, is associated with increased risk for the development of cirrhosis and hepatocellular carcinoma. Although several therapeutic approaches can be used in the context of NAFLD, dietary and physical activities are still the most frequently used strategies. Some pharmacological agents show promising results although no conclusions can be drawn from recent clinical trials. Thyroid hormones [THs; thyroxine (T4) and 3,3′,5-triiodo-L-thyronine (T3)] coordinate a diverse array of physiological events during development and lipid/energy homeostasis and have some potentially therapeutic actions which include inducing weight loss, and lowering plasma cholesterol levels and tissue adiposity. The thyroid hormones exert their physiological effects by binding to specific nuclear receptors [thyroid hormone receptors (TR)] of which the TRβ isoform is liver specific and has been considered a putative target for the treatment of dyslipidemia and fatty liver. In view of this, the aim of the review is (1) to provide an overview of the action of T3 on lipid metabolism with implications for liver steatosis and (2) to provide an update on the current knowledge concerning the administration of TRβ selective thyromimetics (GC-1 and MB07811), as well as of 3,5-diiodo-L-thyronine and its novel functional analogue TRC150094 in animal models of overweight and related disorders including primarily fatty liver.

Key words: Fatty liver; Thyroid hormones; Thyromimetics; 3,5-diiodo-L-thyronine; Lipid metabolism

Core tip: Fatty liver is associated with increased risk for the development of cirrhosis and hepatocellular carcinoma. Thyroid hormones have some potentially therapeutic actions by binding to specific nuclear receptors [thyroid hormone receptors (TR)] of which the TRβ isoform is liver specific and a putative target for the treatment of dyslipidemia and fatty liver. This review provides (1) an overview of the action of T3 on lipid metabolism and (2) an update concerning the administration of TRβ selective thyromimetics (GC-1 and MB07811), as well as of 3,5-diiodo-L-thyronine and its novel functional analogue TRC150094 in animal models of overweight and fatty liver.

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INTRODUCTION

The liver plays essential roles in supporting many meta-

bolic processes and is critically involved in facilitating the maintenance of blood-glucose levels and energy homeostasis. Diet-induced obesity - commonly associated with diseases such as type 2 diabetes (T2DM), hypertension, heart failure, or cancer - also leads to fatty liver or steatosis, a histopathological condition characterized by an excess accumulation within hepatocytes of lipids, which are primarily triglycerides (TGs)[1]. Although the primary metabolic abnormalities leading to lipid accumulation within hepatocytes are still not fully understood, a decreased capacity to oxidize fatty acids, an increased delivery and transport of free fatty acids (FFAs) into the liver as well as an augmented hepatic fatty acid synthesis are likely to play significant roles in the pathogenesis of hepatic steatosis[2-4]. Moreover, steatosis is clearly, inextricably linked to modifications of mitochondrial functions[5,6]. Indeed, the mitochondrion plays an important role in the hepatocyte's metabolism because it is the primary site of fatty acid oxidation and oxidative phosphorylation. Multiple enzymes are involved in mitochondrial β-oxidation, and even partial deficiencies of these enzymes may lead to the development of hepatic steatosis[7,8] (Figure 1A). Two broad categories of hepatic steatosis have been recognized: alcoholic fatty liver disease (AFLD) and nonalcoholic fatty liver disease (NAFLD). In particular, NAFLD, commonly associated with insulin resistance (IR) and cardiovascular diseases[9], comprises a morphological spectrum of liver lesions ranging from simple triglyceride accumulation in hepatocytes (hepatic steatosis) to inflammatory and hepatocellular ballooning injury (non-alcoholic steatohepatitis; NASH), which eventually leads to fibrosis and cirrhosis[1]. The exact mechanism underlying the transition from steatosis to steatohepatitis is still unknown. According to the "two-hit" hypothesis[10]: the first hit involves the accumulation of TGs in hepatocytes that causes a vicious cycle of metabolic dysfunction; once the presence of hepatic steatosis is established, progression to steatohepatitis involves a "second hit" with oxidative stress playing a key role. Fatty liver is more susceptible to oxidative injury[1] and lipid peroxidation[11], and the chemical modification of biological molecules may be directly toxic to the cells or may stimulate host-immune response that leads to inflammation, collagen production and further disease progression[12-14].

Therapeutic interventions in NAFLD are mainly based on lifestyle changes, including diet and exercise[15,16]. Currently, there are no approved pharmacological therapies for NAFLD, but because IR is almost universally present in patients with this condition, drugs that increase insulin sensitivity are currently undergoing extensive evaluation and hold promise as therapeutically effective agents[17,18]. Several other agents, such as antioxidants and hepatoprotective compounds, have been evaluated, and the data was inconclusive or demonstrated no effects[16].

Thyroid hormones [THs; thyroxine (T4) and 3,3',5-triiodo-L-thyronine (T3)] exert a multiplicity of effects and are potent regulators of glucose and lipid metabolism and body weight. In particular, they play an important role in hepatic lipid homeostasis. They exert their physiological effects by binding to specific nuclear receptors, the thyroid hormone receptors (TR) α and β that are widely distributed throughout the body. The β isoform is the major TR expressed in the liver. The beneficial effects of TRβ activation include lowering low-density lipoprotein (LDL) cholesterol, reducing whole body adiposity and weight[19], and increasing the metabolic rate in the liver which could potentially lead to reduced lipid content. However, to date, there is a lack of data available on the specific effects elicited by T3 on liver steatosis. In a recent study, T3 was shown to exert a strong inhibitory effect on the development of steatosis and to cause a rapid regression of fully established steatosis[20].

An excess of thyroid hormone is associated with unwanted effects particularly on the heart (including tachycardia and sudden death) and also on bone and skeletal muscle[21]. Because of these adverse effects of THs, several new TH analogs (generically termed as thyromimetics) have recently been developed to generate effective and safe treatments to counteract obesity and related disorders among which hyperlipidemia and liver steatosis. These either have selective effects on the liver *vs* the heart or bind selectively to TRβ rather than to TRα without cardiac side effects[22]. Such compounds could serve as powerful new tools to address some of the largest medical problems in developed countries-obesity and related disorders[23]. Interestingly, THs also exert non-genomic effects[24] and some are attributable to naturally occurring iodothyronines apart from T4 and T3[25,26]. These THs derivatives are currently being studied to elucidate their potential biological activities and application as anti-hyperlipidemic as well as anti-steatotic agents[22].

This review, including T3 action in the liver and fatty liver, will focus on the current understanding of the actions of thyroid hormone analogues and derivatives in fatty liver in view of the development of potential future therapeutic approaches for the prevention or counteraction of liver steatosis.

ACTIONS OF T3 ON LIPID METABOLISM IN THE LIVER: IMPLICATIONS FOR FATTY LIVER

The pleiotropic effects exerted by T3 includes the maintenance of lipid homeostasis *via* regulation of gene expression in target organs such as liver and adipose tissues. Most T3 effects are mediated by the canonical, or classic, pathway which requires the nuclear T3 receptors[27-29]. Actually, T3 can also signal through non canonical pathways by binding to cytoplasmic or mitochondrial TR isoforms[24]. In mammals, two distinct genes express the TRα and TRβ isoforms. The *TRβ* gene encodes three T3-binding TRβ isoforms (β1, β2, and β3) that share high sequence homology in the DNA and T3-binding domains but differ in length and amino acid sequences in the amino-terminal A/B domain. The *TRα* gene en-

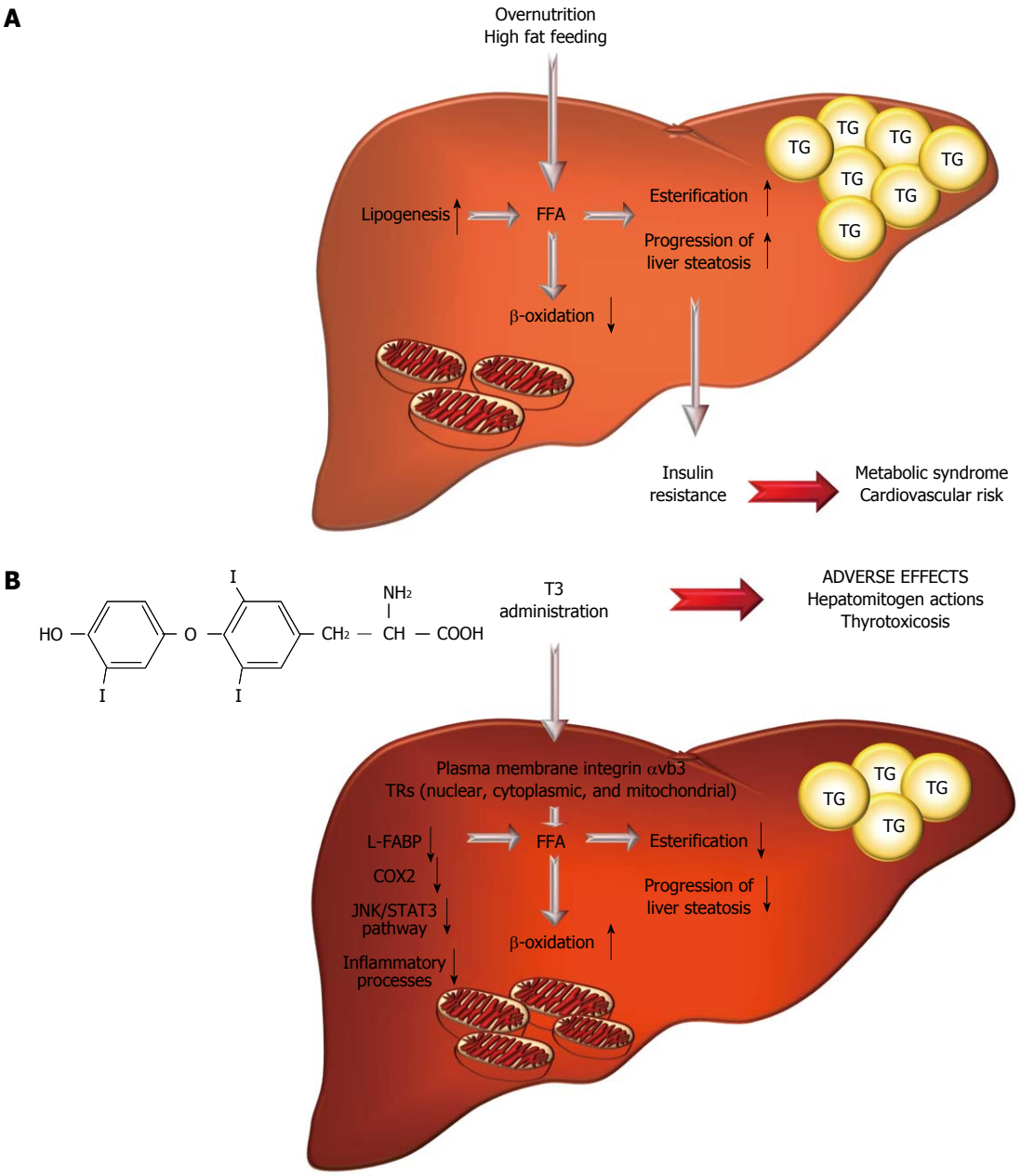

Figure 1 Hepatic lipid partitioning and liver and systemic metabolic damages in nonalcoholic fatty liver disease (A) and a schematic representation of the anti-steatotic effect of 3,3',5-triiodo-L-thyronine (B). A: Hepatic lipid partitioning and liver and systemic metabolic damages in nonalcoholic fatty liver disease. Chronic overnutrition/hyperlipidemic feeding causes fat retention in hepatocytes that, in turn, results in alteration of fat uptake, de novo synthesis (lipogenesis) and oxidation with a significant imbalance of lipid homeostasis. This can subsequently induce insulin-resistance, metabolic syndrome and cardiovascular diseases; B: A schematic representation of the anti-steatotic effect of T3: An update. T3-administration associated adverse effects are also highlighted (for details see the text). T3: 3,3',5-triiodo-L-thyronine; TRs: Thyroid hormone receptor isoforms; FFA: Free fatty acid; TG: Triglyceride; L-FABP: Liver-type fatty acid-binding protein; COX2: Cyclo-oxygenase 2; JNK: c-Jun N-terminal kinases; STAT3: Signal transducer and activator of transcription 3.

codes one T3-high affinity binding TRα1 and two splice variants (TRα2 and TRα3) which differ from TRα1 in length and amino acid sequences in the C-terminal region starting at amino acid 370, and they have no T3-binding activity[30]. While TRα1 is preferentially expressed in the heart, TRβ1 is the major isoform in the liver, kidney and thyroid. However, TRβ2 is predominantly expressed in the brain, adipose tissue and anterior pituitary gland. The liver is an important T3 target tissue[31]. T3 increases the expression of several genes involved in hepatic lipogenesis including fatty acid synthase (FAS), hepatic product spot 14 (which interacts physically and functionally with the TR to regulate malic enzyme gene expression[32]), acyl-CoA synthetase 5, fatty acid transporter protein, malic enzyme, glucose-6-P dehydrogenase (G6PDH)[33], sterol regulatory element binding protein-1c (SREBP-1c)[34]. T3 also induces genes involved in fatty acid oxidation, such as fatty acid transporter (FAT), fatty acid-binding protein (FABP), lipoprotein lipase (LPL)[33], and carnitine palmitoyltransferase-1alpha (CPT-1α), a key rate-limiting enzyme in mitochondrial fatty acid oxidation. In the liver, many of these genes (*e.g.*, malic enzyme, SREBP-1c, FAS

and CPT-1α) are directly regulated by T3/TR as the thyroid hormone response elements (TREs) have been reported in their promoters[34,35]. Importantly, T3 transcriptional activity also depends on several other factors including the type of TREs located on the promoters of target genes, the developmental- and tissue-dependent expression of TR isoforms, and a number of nuclear co-regulatory proteins. TRs bind to TREs not only as homodimers but also as heterodimers with other members of the receptor superfamily, such as retinoic X receptors (RXRs), vitamin D receptor, and all subtypes of the retinoic acid receptors. Heterodimerization with RXR dramatically increases the binding of TRs to TREs, the responsiveness of TR to T3, transcriptional activation[30] and, due to promiscuity of RXR in heterodimerization with many members of the receptor superfamily, allows TR to crosstalk with other receptors. Crosstalk with peroxisome proliferator-activated receptor (PPAR) signaling *via* heterodimerization with RXR by TR is a well-known example[30,36].

Moreover, in the liver, activation of the NAD^+-dependent deacetylase sirtuin 1 (SIRT1) facilitates fatty acid oxidation[37]. Indeed, in hepatocytes isolated from mice lacking SIRT1, fatty acid oxidation rates are reduced, and these mice accumulate lipids within the liver[38]. Recently, SIRT1 has been reported to interact directly with TRβ1, contributing to the T3-mediated stimulation of hepatic genes *via* the activation of several factors such as PPARα, estrogen-related receptor α (ERR-α), and peroxisome proliferator-activated receptor γ coactivator (PGC-1α)[39]. In the liver, PGC1-α promotes expression of genes involved in hepatic fatty acid oxidation[40] and drives the expression of genes involved in hepatic gluconeogenesis *via* interactions with HNF4α (hepatic nuclear factor-4) and FoxO1 (forkhead transcription factor)[41,42]. In particular, PGC-1α appears to be another key player in T3-signaling as it is able to coactivate the TRβ[43] and its overexpression enhances the induction T3-mediated of CPT-1α[44] and PDK4 (pyruvate dehydrogenase kinase, isozyme 4)[45,46]. Interestingly, T3 can also signal in a TRβ-independent manner by binding to surface receptor such as integrin αvb3 receptor, thus activating the MAPK/ERK and PI3K/Akt/mTOR-C1 pathways[24]. Recent studies have highlighted that such effects in hepatocytes have the potential to modulate lipogenesis and cholesterologenesis[47].

Normal serum THs levels are essential for the maintenance of a sufficient pool of cholesterol to meet the body's requirements and to regulate the critical steps of cholesterol synthesis, uptake and metabolism[19]. T3 signaling: (1) stimulates 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG CoA) reductase and farnesyl pyrophosphate (FPP), favoring cholesterol synthesis; (2) up-regulates the LDL receptor (LDLR; low density lipoprotein receptor), increasing cholesterol uptake; and (3) stimulates cholesterol 7α-hydroxylase (CYP7A1), enhancing the metabolism of cholesterol into bile acids[48-50].

Although the T3-mediated actions involved in the regulation of serum lipid homeostasis as well as in hepatic fat metabolism are quite well established, the T3-specific effects on NAFLD are still not fully elucidated. A study using a nutritional model of NAFLD [treating rats with high-fat choline-methionine deficient (CMD) diet] revealed that co-feeding T3 (4 mg/kg of diet) with a CMD diet exerts a strong inhibitory effect on the development of steatosis. Indeed, T3 prevents accumulation of TGs by inducing fatty acid oxidation with subsequent impairment of TGs hepatic synthesis/accumulation, and decreases the expression of liver-type fatty acid-binding protein (L-FABP), an abundant protein in the cytosol of hepatocytes that facilitates fatty acid transport and utilization[20]. Furthermore, the same study showed that T3 administration for only 1 wk, following 10 wk on a CMD diet, caused a rapid regression of fully established steatosis by: (1) dramatically reducing liver TGs levels and cyclooxygenase 2 (COX2) expression; (2) down-regulating pathways, such as JNK (c-Jun N-terminal kinase) and STAT3 (signal transducer and activator of transcription 3) pathways, usually activated in inflammatory processes; and (3) reducing the severity of liver injury as determined by serum levels of transaminases (AST and ALT; aspartate aminotransferase and alanine aminotransferase)[20] (Figure 1B).

Of note, T3 is also a potent mitogen that, in the liver, induces Cyclin-D1 expression[51]. Very recently, it has been shown that it exerts hepatocyte mitogenic response by PKA-dependent β-catenin activation, thus eliciting a potent liver regeneration action[52]. This has suggested that T3 can have therapeutic relevance in the treatment of selected cases of hepatic insufficiency.

However, long term treatment with T3, both in animals and humans, can produce several adverse effects including systemic thyrotoxicosis. Thus, extensive research is dedicated to the identification of new effective and safe active molecules (T3-derivatives and analogues) for the treatment of dyslipidemias, NAFLD, obesity and related disorders.

In particular, the development of synthetic thyroid hormone analogues which have tissue-selective hormone actions (*i.e.*, selective thyromimetics) has been pursued.

THYROMIMETICS AND LIVER STEATOSIS

As mentioned previously, many of the T3 actions are tissue-specific and are primarily mediated by a panel of TR isoforms that are expressed in different ratios in various tissues. Thus, there is a rationale to pursue approaches that selectively modulate TRs function, and several agents have been shown to have some β-selective, hepatic selective and/or cardiac sparring activities. The possibility of selectively targeting the TRβ was suggested by the findings that the TRα-forms may preferentially regulate the heart rate, whereas many other actions of T3 are mediated by the TRβ. X-ray crystal structures of the TRα and TRβ ligand-binding domains (LBDs) suggested that a

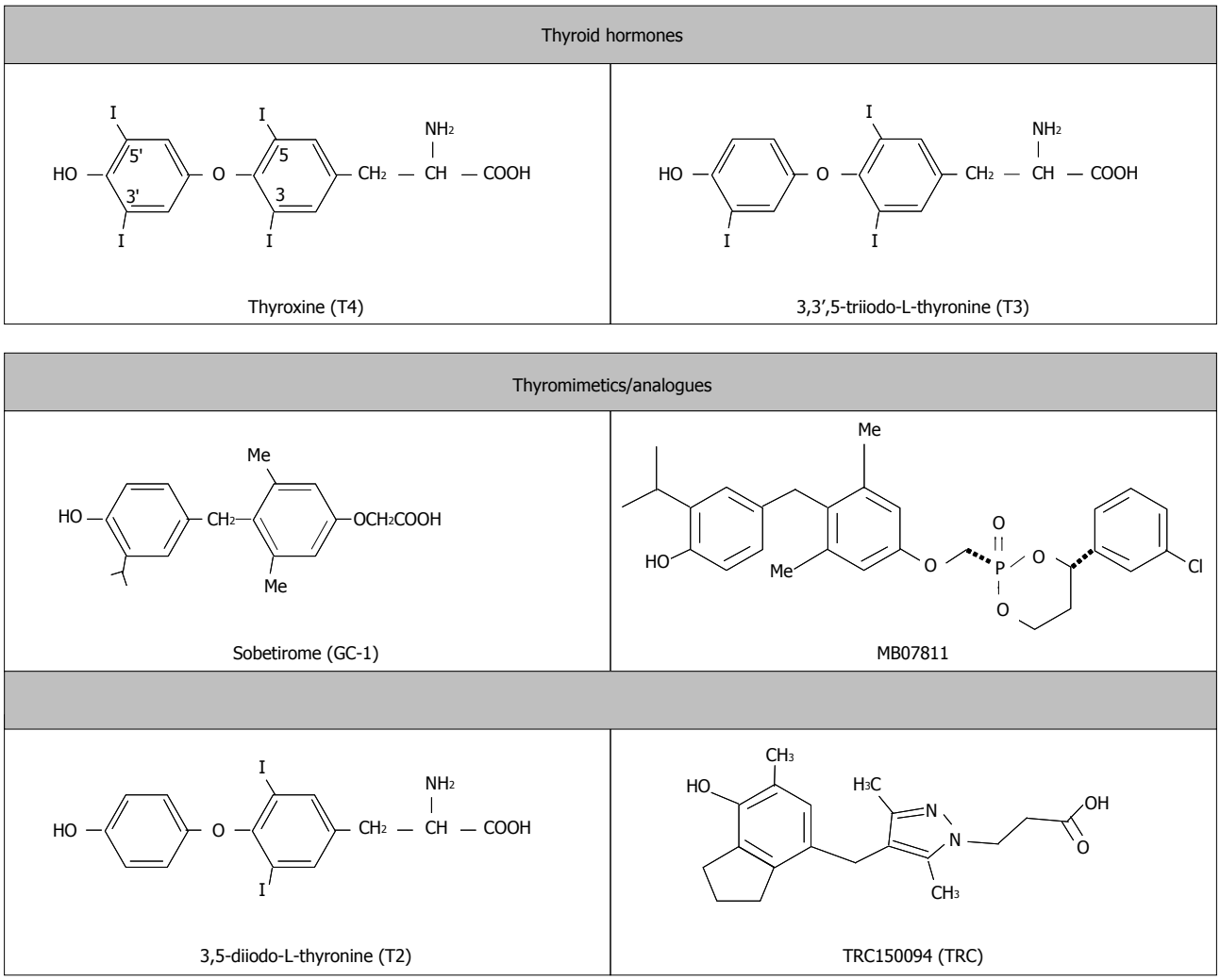

Figure 2 Chemical structure of thyroid hormones and thyromimetics/analogues with reported anti-steatotic effects.

single amino acid difference in the ligand-binding cavities of the two receptors could affect hydrogen bonding in the receptor region where the ligand's 1-position substituent fits and might be exploited to generate β-selective ligands[53].

The development of a TRβ-selective agonist has prompted a number of studies addressing whether such molecules could be used to trigger the metabolic effects of T3 while preserving the TRα-expressing tissues[54-58]. Essentially, these studies have been encouraging as it has been shown that the use of TRβ-selective agonists can prevent or improve metabolic parameters and/or complications resulting from high-fat feeding, NAFLD[20], or genetic hypercholesterolemia[59,60] with the liver being their major target. Indeed, tissue distribution analyses suggest that these molecules achieve TRβ selectivity by virtue of being concentrated predominantly in liver[55,61]. TRβ activation in the liver also favorably affects plasma cholesterol and lipoprotein levels by multiple mechanisms, which include increasing: (1) LDL clearance through increased expression of LDLR, (2) high-density lipoprotein (HDL) uptake through SR-B1 (scavenger receptor class B type 1); and (3) bile acid synthesis *via* CYP7A1[62].

To exploit the favorable consequences of hepatic TRβ activation, a variety of synthetic TRβ agonists have been prepared and tested on a variety of experimental models[63-73]. Ideally, these selective TRβ agonists, would cause modest increases in the metabolic rate without tachycardia[22]. However, it has been reported that most of thyromimetics could suppress thyroid axis and lower serum T4/T3 levels, especially at high doses.

Here, we will discuss two thyromimetics, namely GC-1[74] and MB07811[57,75,76] (Figure 2), for which these effects are less marked and which, at the same time, elicit anti-steatotic effects. In particular, MB07811, being specifically targeted to the liver, reduces serum T4 levels (-50% in Sprague-Dawley rats) probably by enhancing T4 to T3 conversion through deiodinase 1[53,57]. As far as it concerns GC-1, it has been shown a dose-dependent ability to reduce thyroid-stimulating hormone (TSH) levels, being this action 20-fold less potent than that of T3[55].

GC-1 (or sobetirome) is a halogen-free thyroid hormone agonist[74]. Although the structural changes it contains with respect to the natural hormone T3 (*i.e.*, replacement of the three iodines with methyl and isopro-

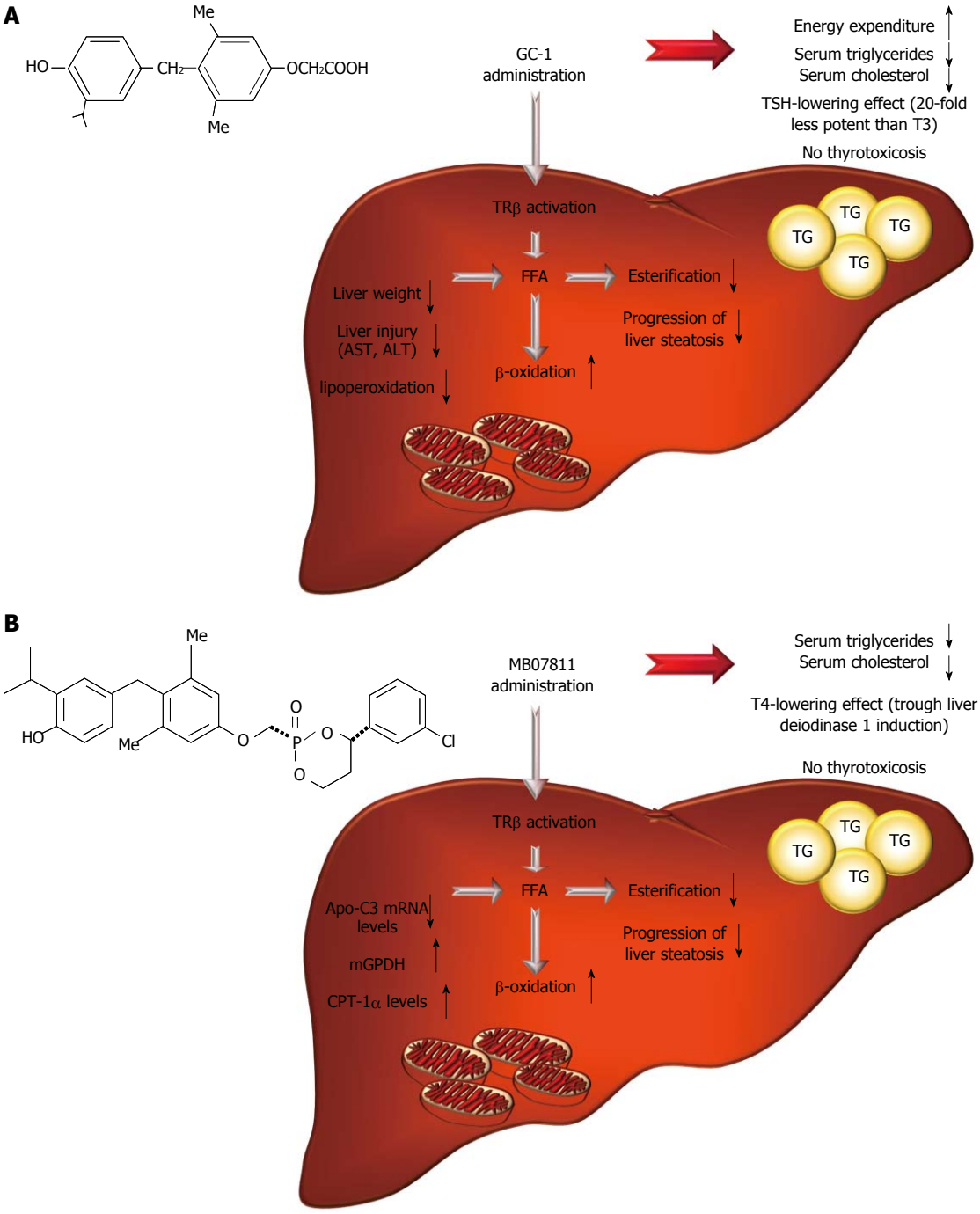

Figure 3 A summary of key events and molecular pathways underlying GC-1 (A) and MB07811 (B) anti-steatotic and hypolipidemic effects (for details see the text). TSH: Thyroid-stimulating hormone; T3: 3,3',5-triiodo-L-thyronine; TRβ: Thyroid hormone receptor β isoform; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; FFA: Free fatty acid; TG: Triglyceride; T4: Thyroxine; Apo-C3: Apolipoprotein C3; mGPDH: Mitochondrial glycerol-3-phosphate dehydrogenase; CPT-1α: Carnitine palmitoyltransferase-1α.

pyl groups, replacement of the biaryl-ether linkage with methylene linkage, and replacement of the amino acid side chain with an oxyacetic acid side chain)[77], it binds the TRβ with an affinity that is comparable to that of T3[22]. Functionally, when GC1 is administered to rats undergoing a CMD diet prevents and reverses the hepatic steatosis much like T3[20]. Moreover, similar to T3, GC-1 can reduce liver weight, liver weight/body weight ratio, and serum TGs levels. GC-1 also causes a reduction of CMD-induced TGs accumulation in the liver, with the disappearance of hepatic TGs being accompanied by a concomitant decrease of lipoperoxidation, and of liver injury as indicated by the significant reduction in AST and ALT levels[20]. These findings made GC-1 an ideal molecule for therapies against fatty liver disease. Interestingly, GC-1 also stimulates energy expenditure[78] and mitochondrial oxidative processes but to a lesser extent compared to T3[79,80] (Figure 3A). Notably, animal studies[55] revealed that treatment with GC-1 also induces a reduction of cholesterol levels similar to that obtained

with equimolar doses of T3 and even higher than that achieved with the most common drugs currently available on the market for the treatment of hypercholesterolemia, such as the inhibitors of HMG CoA reductase (statins)[77]. In doing so, GC-1 regulates key steps in the reverse cholesterol transport pathway[62], increasing the expression of HDL receptor SR-B1 in the liver, stimulating the activity of CYP7A1 and inducing the expression of hepatic ATP-binding cassette proteins G5/G8 (ABCG5/G8), which promote biliary cholesterol secretion. Consequently, treated animals displayed an increased turnover of plasma HDL cholesterol, and an increased amount of fecal excretion of bile acids and cholesterol[70]. Phase 1 clinical studies tested the therapeutic concept of lowering cholesterol and found GC-1 to be generally well tolerated at all doses studied[81,82].

In a recent study, GC-1 has also been shown to be capable of markedly reducing serum cholesterol in mice devoid of functional LDLRs by inducing CYP7A1 expression[60]. These results, having elucidated the possibility that a LDLR-independent mechanism could underlie GC-1 action, potentiated the idea that GC-1 may represent a promising cholesterol-lowering therapeutic with a specific application for the treatment of diseases such as homozygous familial hypercholesterolemia. Currently, there are only limited treatment options for this disorder because most therapeutics are only minimally effective.

Another agent being studied is MB07811 which exhibits increased TR activation in liver relative to other tissues[57]. By using several experimental approaches, MB07811 was shown to have anti-steatotic activity and was able to reduce hepatic triglyceride levels in both normal and metabolically-challenged animal models, including ob/ob mice, Zucker rats, and mice with diet-induced obesity (rodent models of NAFLD)[83]. The main mechanism underlying MB07811 effects appears to be an augmented metabolic rate in the liver and, specifically, an increased rate of mitochondrial β-oxidation. MB07811 increases: (1) the levels of CPT-1α[35] and short and intermediate length acyl-carnitine species in plasma and (2) the liver mitochondrial respiration rates as well as the activity of hepatic mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH), an enzyme which is important for energy production and dissipation. Decreased mRNA levels of apolipoprotein C3 (Apo-C3), an inhibitor of hepatic lipase activity, might also contribute to the activation of fatty acid oxidation pathways. Additionally, MB07811 can also lower both serum cholesterol and triglyceride levels[83,84] (Figure 3B). These data in rodents confirm that MB07811 represents a novel class of liver-targeted TR agonists with beneficial LDL-lowering properties, and suggest that these compounds may provide additional therapeutic benefit to hyperlipidemic patients with concomitant NAFLD[83]. The human Phase 1b clinical trial showed reduced LDL cholesterol and TGs levels in both normolipidemic and hyperlipidemic subjects without severe adverse events[23].

Together, these data demonstrate that selective activation of hepatic TR prevents or reverses fatty liver and reveals a new approach to treat NAFLD based on selectively burning hepatic fat.

Now, the question is whether other molecules such as naturally occurring TH metabolites, even deprived of TR selectivity or characterized by low binding affinity for TRs, can have uses as therapeutic applications.

NATURALLY OCCURRING IODOTHYRONINES AND LIVER STEATOSIS: 3,5-T2

An increasing amount of data indicates that there are at least four natural iodothyronines with significant, but not identical, biological activities, namely T4, T3, rT3 (reverse T3), and 3,5-diiodo-L-thyronine (T2). T2 is particularly intriguing because of its effects on metabolism[22,25,26]. T2 has been estimated, in euthyroid rats, to reach serum concentrations of approximately 5 pM and is present in liver at concentrations of approximately 1.0 fmol/100 mg[85]. In humans, serum T2 levels consistently elevated in disease states, with the mean T2 serum level being 16.2 ± 6.4 pM in healthy subjects, 21.6 ± 4.8 pM in patients with brain tumors and 46.7 ± 48.8 pM in patients with sepsis[86].

T2 measurements are routinely taken using methods based on immunoassays, an approach with high sensitivity but which lacks specificity for many analytes[87,88]. In recent years, mass spectrometry (MS) techniques have drawn attention to the analyses of T4 and T3 because they provide high mass accuracy, structural information, and have the ability to quantify the hormones[89-93]. A recently developed methodology revealed that electrospray ionization tandem mass spectrometry (ESI-MS/MS) can be used for identification and quantification of mixtures of isomers and has been applied to identify and quantify T3 and rT3 isomers as well as T2 isomers[94,95]. Currently, however, intrinsic instrumental limits restrain the application of such approaches as routine tools for biological samples analysis and slow the advancements in understanding T2 metabolism[96,97].

T2 is a product of a currently unknown peripheral enzymatic process most probably utilizing T3 as its precursor[85] and has 50-1000 times lower affinity for TR than T3[98]. Thus, it is unlikely that TR activation represents a central mechanism in its effects on metabolism, at least in physiological conditions. However, more recent studies have reported new data concerning T2 binding to TRβ isoforms in teleosts[99,100]. Specifically, T2 has been shown to bind and transactivate both the human and the long tilapia TRβ1 isoform whereas T3 preferentially binds the short isoform. These results prompted a reevaluation of the mechanisms of action of thyroid hormone metabolites.

Several studies on T2 effects in mammals revealed its ability to stimulate cellular/mitochondrial respiration by pathways with mitochondria and bioenergetic mecha-

nisms being the major targets[26,80,101-103]. Outside the mitochondria, T2 also has effects on carriers, ion-exchangers, and enzymes, and may affect the transcription of some genes, but again the underlying mechanisms appear to be different from those elicited by T3[26].

In 1998, Arnold *et al*[104] identified the Va subunit of the mitochondrial respiratory chain complex cytochrome-*c* oxidase (COX) as a specific binding site for T2 using photoaffinity labeling procedures. T2 binding to the COX complex abolishes the allosteric ATP inhibition of COX which leads to a decrease in the respiratory control ratio of the complex[105] thus rendering the oxidative phosphorylation more inefficient.

The biological and pharmacological importance of T2 has become a topic of considerable interest to researchers during the past few years, and now represents a significant and promising issue in the field of metabolism and THs.

The effects and mechanisms underlining the beneficial actions of T2 have so far been studied with both *in vivo* and *in vitro*[106-108] models. *In vivo* studies from different laboratories have shown that acute or chronic administration of T2 to rats results in significant changes in mitochondrial activities and resting metabolic rate[22,26,109].

A recent study reported an increased basal metabolic rate and decreased body weight also in humans chronically administrated with T2 with no deleterious side effects on the thyroid axis or at the cardiac level[110].

Of the currently described *in vivo* stimulatory and beneficial effects of T2, a particular physiological and pharmacological relevance appears to be associated with those effects that we can define as hypolipidemic and anti-steatotic effects which have been described in several animal models[111-114]. Among the methods to study liver metabolism and physiology, the variation in the nutritional status is a widely used approach because of its ability to affect several signaling pathways and regulatory mechanisms[115-118]. High fat feeding (HFD) in animals, in particular, has the advantage to mimic most features of human fat overload and overnutrition and allows the study of obesity and related disorders such as ectopic fat accumulation.

Specifically, the administration of T2 to rats subjected to HFD is able to prevent and reduce the visceral fat accumulation as well as hepatosteatosis, serum levels of triglycerides and cholesterol, and the onset of IR without inducing thyrotoxicosis[111,112,114,119]. Moreover, T2 has been reported to elicit additional beneficial effects on lipid metabolism by reducing LDL-cholesterol in a LDLR independent way in a mouse model of familial hypercholesterolemia[120].

The simultaneous administration of T2 (25 μg/100 g body weight) to rats receiving a HFD for 4 wk can prevent liver steatosis by stimulating hepatic fatty acid oxidation and increasing mitochondrial uncoupling[111]. This leads to a less efficient utilization of lipid substrates, and helps to prevent body-weight gain, hepatic fat accumulation, hypertriglyceridemia and hypercholesterolemia levels without inducing changes in T3 and T4 serum levels or affecting the hypothalamus-pituitary-thyroid (HPT) axis[111,119].

The T2 effects on liver fatty acid oxidation are paralleled by an increased entry of activated fatty acids into the mitochondria *via* activation of the CPT system. This leads to a strong reduction in the fatty acids inside the cell and a strong activation of AMP-activated protein kinase (AMPK), enhancing the cycle of fat uptake and fat burning and the disappearance of lipid droplets (LDs)[111].

Importantly, T2 administration prevents the HDF-induced lipid peroxidation, as well as the increase in H_2O_2 metabolism counteracting both lipid accumulation and oxidative stress associated with increased fat metabolism[121].

In a more recent study, to further investigate how T2 affects lipid and glucose metabolism in HFD rats eliciting beneficial effects on liver, independently of AMPK, the involvement of another important regulator of metabolic balance[122-127], SIRT1, was studied[114]. In HFD rats, T2 was demonstrated to (1) rapidly increase hepatic nuclear SIRT1 activity and (2) through SIRT1-activation, deacetylate PGC-1α and SREBP-1c with a concomitant upregulation of genes involved in mitochondrial biogenesis and downregulation of lipogenic genes[114]. Moreover, the obtained data added new information on the time-latency of the anti-steatotic effect of T2 which within 6 h after administration, rapidly and directly activates hepatic SIRT1 (affecting β-oxidation and mitochondrial biogenesis) and later (4 wk) promotes AMPK phosphorylation/activation thus profoundly modulating liver expression pattern of genes and proteins.

A proteomic study[113] showed that the steatotic effect of HFD, and the anti-steatotic effect of T2-treatment are strictly associated with altered expression levels of several proteins and enzymes involved in key liver metabolic (canonical and non-canonical) pathways across different subcellular compartments (*i.e.*, cytoplasm, mitochondria and nuclei). These pathways included: fatty acid metabolism, ketone-bodies and energy metabolism, amino acid and nitrogen metabolism, the urea cycle and the stress response and protein turnover.

All the analyzed liver subcellular compartments were significantly affected, in terms of protein expression, by both HFD and long-term T2-treatment. However, mitochondria appeared to be a major target for the metabolic and energy adaptations induced by fat-overload, and displayed a significant response, in terms of their proteome, to T2-treatment. These data supported the concept that T2-supplementation while having hypolipidemic and anti-steatosis effects, may provide protection against diet-induced liver damage, possibly by counteracting the alterations in the expression of several cellular proteins, reducing oxidative stress and impairing the mitochondrial respiratory chain[113].

T2 administration to rats is also able to reduce pre-existing hepatic fat accumulation (that had already been induced by feeding with a HFD)[112] by eliciting systemic

and tissue specific effects. In particular, T2, without suppressing TSH, decreases body weight gain, metabolic efficiency and serum levels of cholesterol, triglycerides and ALT. In the liver, T2 increases hepatic mitochondrial oxygen consumption and fatty acid oxidation and activates mitochondrial proton leak reducing mitochondrial oxidative stress[112].

In vitro studies have been performed to address whether the anti-steatotic effect of T2 is due to the direct action of T2 on the liver or if it is a secondary effect due to upstream changes in endocrine or metabolic pathways. Primary cultures of rat hepatocytes overloaded with lipids ("fatty hepatocytes") and then treated with T2 showed a reduction in: (1) lipid content and LD diameter; (2) PPARs expression; and (3) activities of acyl-CoA oxidase (AOX) and antioxidant enzymes. These data support a direct role of T2 in reducing the excess fat in cultured hepatocytes[128]. The putative involvement of TRs in mediating such lipid-lowering effects of T2 has been elucidated using the rat hepatoma FaO cellular model, which is defective for functional TRs[128]. The addition of T2 to lipid-overloaded cells resulted in: (1) reduction in lipid content; (2) downregulation of PPARα, PPARγ, and AOX expression; (3) increase in PPARδ expression; and (4) stimulation of mitochondrial uncoupling[128]. These data demonstrate, for the first time that the *in vitro* lipid-lowering actions of T2 may be not mediated by TRs.

All the utilized approaches, both in animal models and humans, successfully highlighted metabolic actions and potential pharmacological use of T2. Notably, T2, without thyrotoxic side effects, increasing resting metabolic rate, decreasing adiposity and body weight, could be considered an active agent in preparation for treatment of metabolic disorders, such as T2DM, overweight and NAFLD (Figure 4A).

THYROID HORMONE FUNCTIONAL ANALOGUES: TRC150094 (TRC)

T2 research has been recently focused on the discovery and development of T2 functional analogues with therapeutic potentials. In particular, a series of novel substituted pyrazoles were designed and synthesized as T2 analogs with lower affinities toward TRs. Among these molecules, TRC150094 (TRC) has attracted particular attention emerging as a novel thyromimetic and functional T2-analogue linking fat consumption with the pathogenesis of hepatic steatosis. The chemical name for TRC is 3-[4-(7-hydroxy-6-methyl-indan-4-ylmethyl)-3,5-dimethyl-pyrazol-1-yl]-propionic acid, and its chemical structure is shown in Figure 2. TRC was synthesized by the Torrent Research Centre, Torrent Pharmaceuticals Ltd., Ahmedabad, Gujarat, India and has a much lower potency toward both TRα1 and TRβ1 isoforms' activation than T3. Therefore, it is devoid of adverse effects on the heart classically associated with TR hyperactivation [the rank order of potencies for TR (α1/β1) transcriptional activation being T3 > T2 >>TRC[129]].

When screened for its *in vivo* metabolic effects, TRC (injected, at a dose of 0.750 mg/100 g body weight, in rats simultaneously undergoing a HFD for 4 wk) counteracts the hepatic pathological condition observed in HFD rats without any thyrotoxic effects[129]. The anti-steatotic activity of TRC is due to increased rates of mitochondrial fatty acid uptake and oxidation, respiratory chain activity and resting metabolic rate, and to the activation of the CPT system[129,130]. Importantly, TRC significantly increases SIRT1 activity although the SIRT1 protein level remains unaltered. These results strongly suggested that a TRC-induced increase in SIRT1 activity might underlie the increase in fatty acid oxidation and the prevention of liver steatosis observed in the TRC-treated rats[129].

An integrated functional study performed by combining *in vivo* and *ex vivo* metabolic assays with proteomic and bioinformatic analyses showed that TRC administration to rats with pre-existing body fat accumulation significantly altered the expression levels of several proteins and enzymes involved in key liver metabolic pathways, including amino acid, nitrogen, fructose and mannose metabolism, and RXR activation and function[130]. Consistent with the increased oxidation of mitochondrial fatty acids and the unaltered mitochondrial efficiency, numerous mitochondrial enzymes associated with fatty acid oxidation and energy metabolism were increased in livers from HFD-TRC[130].

Oral administration of TRC to obese Zucker rats (obese ZSF1, spontaneously hypertensive fatty rats) decreased hepatic steatosis[131] by inducing a significant increase in mitochondrial respiration as well as an increased fatty acids oxidation. All the above mentioned studies have so far provided the characterization of the pharmacological/metabolic effects of TRC and highlighted its potential utility as a new compound with effectiveness for prevention/amelioration of certain key biochemical parameters altered during feeding on a HFD-regimen and a cluster of multiple cardiovascular risk factors associated with visceral obesity, steatosis and metabolic derangements (Figure 4B). If reproduced in humans, these results could determine whether TRC150094 represents an attractive therapeutic agent for the treatment of overweight dysglycemic patients.

Indeed, clinical trials are currently in progress to translate these effects into approaches for the treatment of human obesity.

THYROID HORMONES AND MITOCHONDRIAL FUNCTIONS

As already stated, mitochondria play a fundamental role in the development, perpetuation and worsening of liver steatosis and NAFLD. Indeed, the generation of NAFLD, apart from involving defects or polymorphisms in mitochondrial DNA, can be an important consequence of damages to the respiratory chain complexes impairing mitochondrial oxidative capacity, particularly critical when fatty acid supply to the hepatocytes is increased as

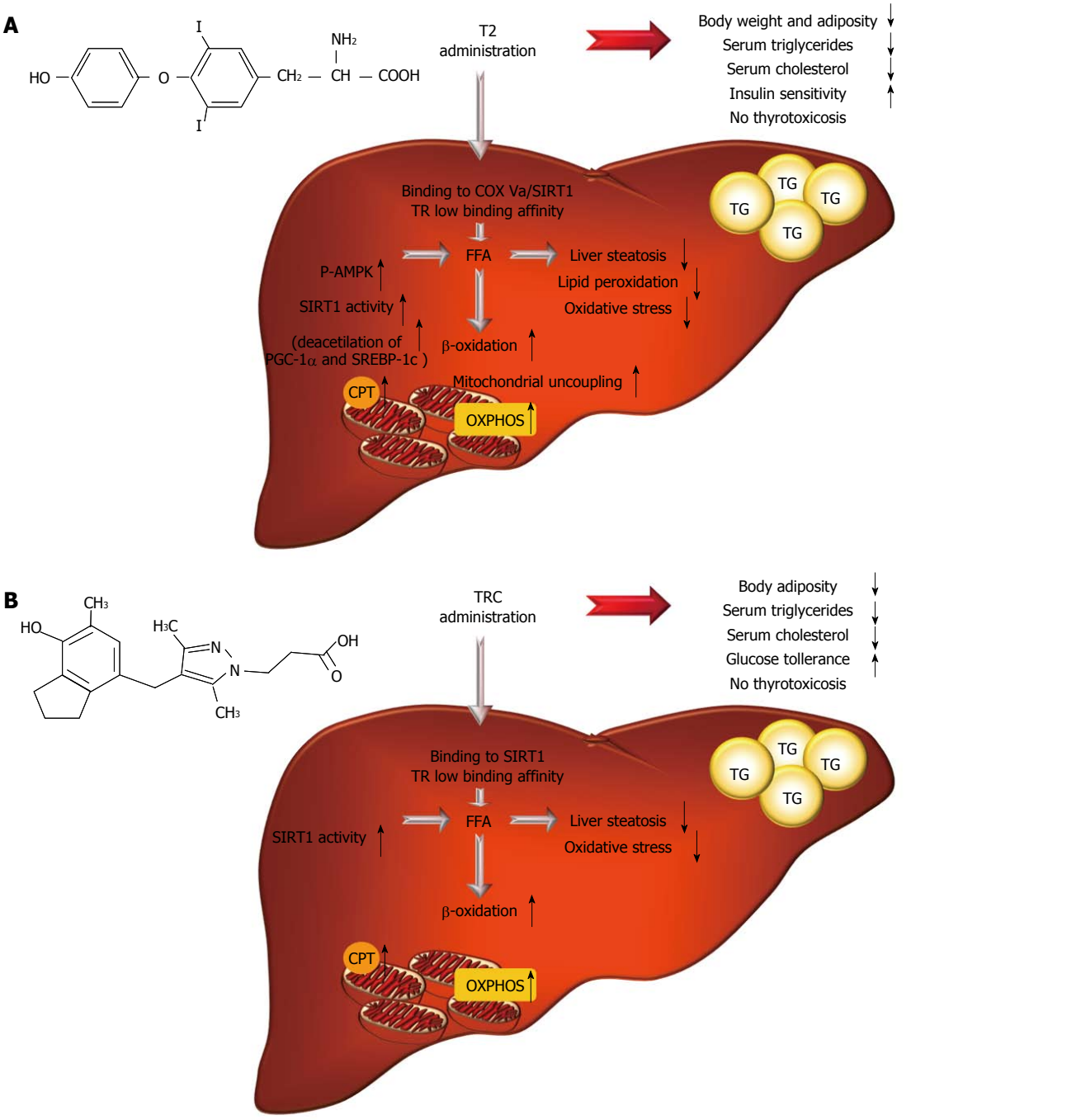

Figure 4 A summary of key events and molecular pathways underlying T2 (A) and TRC (B) anti-steatotic and hypolipidemic effects (for details see the text). T2: 3,5-diiodo-L-thyronine; TR: Thyroid hormone receptor; COX Va: Cytochrome-*c* oxidase Va subunit; FFA: Free fatty acid; TG: Triglyceride; P-AMPK: Phosphorylated AMP-activated protein kinase; SIRT1: NAD^+-dependent deacetylase sirtuin 1; PGC-1α: Peroxisome proliferator-activated receptor γ coactivator; SREBP-1c: Sterol response element binding protein-1c; CPT: Carnitine palmitoyltransferase system; OXPHOS: Oxidative phosphorylation system.

in calorie-rich diets[132].

Mitochondrial bioenergetics deficits may be the consequence of: (1) a progressive decay of oxidative capacity with impairments of β-oxidation; and (2) major changes in the redox balance with increased reactive oxygen species production.

It is widely recognized that mitochondria are central targets for THs actions. Indeed, mitochondria, providing about the 90% of the cellular energy supply, likely may be a major player of the so called calorigenic effects of THs[133]. In particular, T3 stimulates mitochondriogenesis and thereby augments cellular oxidative capacity and induces, at the same time, substantial modifications in mitochondrial inner membrane protein and lipid compositions, activating uncoupling of oxidative phosphorylation[133].

In terms of time latency, two types of effects of T3 on mitochondria have been described: (1) a rapid stimulation of respiration, which is evident within minutes/hours after hormone treatment; (2) a delayed induction

of mitochondrial biogenesis and changes in mitochondrial mass, which occur one to several days after hormone treatment. The first effect is probably due to extranuclear/non-genomic mechanisms; the second one involves both T3-responsive nuclear genes and a direct action of T3 at mitochondrial level[109,133]. In other words, this second effect allows T3 to modulate mitochondria activity in two different ways: direct or indirect. The direct action requires the presence inside the organelles of specific binding sites for the hormone[134-136] while the indirect one possibly requires T3 binding to extramitochondrial sites and the modulation of the expression of either nuclear-encoded mitochondrial proteins or intermediate factors (*e.g.*, nuclear respiratory factors 1 and 2; mitochondrial transcription factor A)[137,138].

T3, by the regulation of these pathways, allows the coordinated expression of both the nuclear and the mitochondrial genome that in turn modulates mitochondrial biogenesis, turnover and bioenergetics.

Although the network of factors and cellular events involved in T3 signaling remains incompletely understood, the so far described mechanisms can justify, at least in part, the above reported hypolipidemic and antisteatotic effects elicited by T3 in NAFLD.

CONCLUSION

Considering the impact of THs on the maintenance of lipid homeostasis but also of their adverse effects in TH excess states, recent efforts to identify effective and safe treatments for the counteraction of metabolic disorders (such as liver steatosis), has led to the development and characterization of thyromimetics. Some of these analogs are undergoing further examinations for possible clinical applications. However, despite their original promise, it is unlikely that any first generation synthetic ligands (*i.e.*, GC-1 and MB07811) which already reached human clinical trials will develop into therapeutics. Thus, attention should be focused on other molecules, such as T2 or TRC which could have therapeutic applications. Indeed, such compounds could serve as powerful new tools to address some of the largest over-nutrition associated medical problems as they are able to reduce, at least in animal models of diet-induced obesity, body adiposity, serum triglycerides and cholesterol. They also preserve glucose homeostasis without thyrotoxic side effects. Notably, the hypolipidemic effect of T2 is associated with a potent ability in both preventing and reducing fatty liver. Increasing evidence supports TH derivatives and analogues as attractive active agents that could be taken into consideration for the establishment of new treatments in the counteraction of metabolic disorders, such as T2DM, obesity and NAFLD, thus clinical trials are desirable.

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MINIREVIEWS

Management of gastric variceal bleeding: Role of endoscopy and endoscopic ultrasound

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Abstract

Gastric varices (GVs) are notorious to bleed massively and often difficult to manage with conventional techniques. This mini-review addresses endoscopic management principles for gastric variceal bleeding, including limitations of ligation and sclerotherapy and merits of endoscopic variceal obliteration. The article also discusses how emerging use of endoscopic ultrasound provides optimism of better diagnosis, improved classification, innovative management strategies and confirmatory tool for eradication of GVs.

Key words: Gastric; Varices; Endoscopy; Ligation; Sclerotherapy; Management; Transjugular intrahepatic portosystemic shunt; Endoscopic ultrasound; Balloon-occluded retrograde transvenous obliteration; Endoscopic variceal obliteration

Core tip: This mini-review addresses endoscopic management principles for gastric variceal bleeding. Endoscopic variceal obliteration (EVO) with tissue adhesives is the currently accepted strategy for controlling bleeding and eradicating gastric varices (GVs). EVO is deemed better than both variceal ligation and sclerotherapy in randomized controlled trials. One unsettled issue with EVO is if routine reinjection is better than reinjection in case of rebleeding. The experience with combination treatments is still premature. For secondary prophylaxis, EVO, transjugular intrahepatic portosystemic shunt or beta-blocker use is recommended. Emerging use of EUS provides optimism of better diagnosis, improved classification, innovative management strategies and confirmatory tool for eradication of GVs.

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INTRODUCTION

The natural history of gastric varices (GVs) is less understood than that of esophageal varices (EVs). GVs may be seen in 18%-70% of the patients with portal hypertension (PHT) and are probable source of bleeding in 10%-36% of patients with acute variceal bleeding (AVB)[1-3]. Isolated GVs (IGVs), without EVs, are seen in 5%-12% of patients with PHT[1-3]. They are also commonly seen in patients with non-cirrhotic portal hypertension (NCPHT),

especially with splenic vein thrombosis (SVT). They are more commonly associated with shunts than EVs, most commonly spleno-renal shunt, and their management is quite different from that of EVs.

CLASSIFICATIONS

GVs are commonly classified according to Sarin's classification[4,5] based on location and direction of blood flow: GOV1 (Gastro-Oesophageal Varices) are the most common (74% of all GV) and consist of the esophageal varices extending along the lesser curvature of stomach; GOV2 are the extension of the esophageal varices along the greater curvature near the fundus; IGV1 are isolated gastric varices localized to fundus, without any associated esophageal varices. These arise from spleno-renal or gastro-renal shunts where the feeding vessel arises from the splenic hilum and drains in to left renal vein through gastric cardia/fundus veins. GOV2 and IGV1 are sometimes together called "fundic varices". IGV2 are the isolated gastric varices present elsewhere other than the fundus, which drain in a similar fashion into left renal vein but with multiple tributaries. It is reported that fundal varices (GOV2 and IGV1), though less common than GOV1 varices, are noted to account for 80% of patients with bleeding GV.

Hashizume *et al*[6] proposed an alternate classification of GVs based on endoscopic findings, taking into account their shape (tortuous = F1, nodular = F2, and tumorous = F3), location (anterior = La, posterior = Lp, lesser curvature = Ll, greater curvature = Lg of the cardia, and fundic area = Lf) and color (white = Cw or red = Cr) and further emphasized on presence of glossy, thin-walled focal redness on the varix called as red color spot (RC spot) as a marker of impending bleeding risk[6].

BLEEDING RISK OF GVS

Although GVs are known to bleed less frequently than the EVs, however when they do, they bleed massively and are difficult to achieve primary hemostasis, with a mortality rate of 10%-30%[4,5]. Their chance of re-bleeding is high (35% to 90%) after spontaneous remission and 22%-37% with the glue technique[4,5]. The chance of variceal bleeding is driven by the pressure changes rather than hemostatic forces. The pressures in the GVs are lower than the in the EVs because of their larger size and more frequent presence of the shunts like spleno-renal[7,8]. Despite this, their rupture is more devastating because of the fact that the wall stress increases dramatically even with small rise in the portal pressures due to their larger radius. When there is increase in transmural pressure, the variceal size increases and wall thickness decreases, which leads to rupture[7,8]. The factors which predict hemorrhage in EVs also govern GVs: most importantly the size of the varices (15% in patients with large varices, which are defined as > 10 mm), decompensated cirrhosis and endoscopic presence of the red wale sign. Another factor implicated in increase in incidence and/or size of fundic varices and possible bleeding is the treatment of EVs by either endoscopic variceal ligation (EVL) or endoscopic sclerotherapy (EST)[9]. The plausible explanation is that after treatment the existing collaterals are not sufficient enough to decompress the portal pressure causing an increased incidence of fundic varices.

GENERAL PRINCIPLES OF MANAGEMENT OF BLEEDING GVS

The preliminary management of bleeding GVs is the same as any other variceal bleeding[1-3]. Fluid resuscitation, airway protection, antibiotic administration for the bacterial peritonitis prophylaxis and use of vasoactive agents like octreotide and acid suppressant agents like proton pump inhibitors form cornerstone of initial management. Cautious administration of the blood products (to achieve a target of hemoglobin level between 7-8 g/dL) is advocated as there is potential risk of increased re-bleeding if the portal pressures increase due to repeated transfusions. A schematic of management algorithm of GVs is presented in Figure 1.

Treatment options for acute GV bleeding are varied and include medical, surgical, endoscopic, and endovascular approaches[1-3]. Two general methods exist to deal with bleeding GVs: directly exclude the varices from the porto-systemic system or indirectly decrease the pressure in the varices by decompressing the portal system.

Direct approach

Variceal management by direct endoscopy or endoscopic ultrasound.

Role of endoscopy: Once the patient is deemed stable from airway and circulation standpoint, an esophagogastroduodenoscopy (EGD) should be performed, which might show active bleeding or reveal stigmata of recent bleeding, in addition to qualify type of GVs and concomitant presence of EVs or PHG[1-3].

Several endoscopic techniques have been tested to control acute gastric variceal bleeding with varying successes. However, the universal phenomena is that majority of the methods used in controlling the bleeding EVs are difficult to practice in GVs and are inconsistently successful. These include endoscopic injection sclerotherapy (EIS) and esophageal variceal ligation (EVL). The varying success of these methods may be owing to different physiology and size of GVs which pose technical problems.

GV ligation: The main indications for ligation in management of acute GV bleeding is banding of GOV1 varices, which are extensions of EVs into the stomach along the lesser curvature or as salvage strategy if other modalities are not available[10]. Studies suggest good hemostasis efficacy and comparable re-bleeding rates of GOV1 ligation to EVL of EVs. There is limited role for ligation

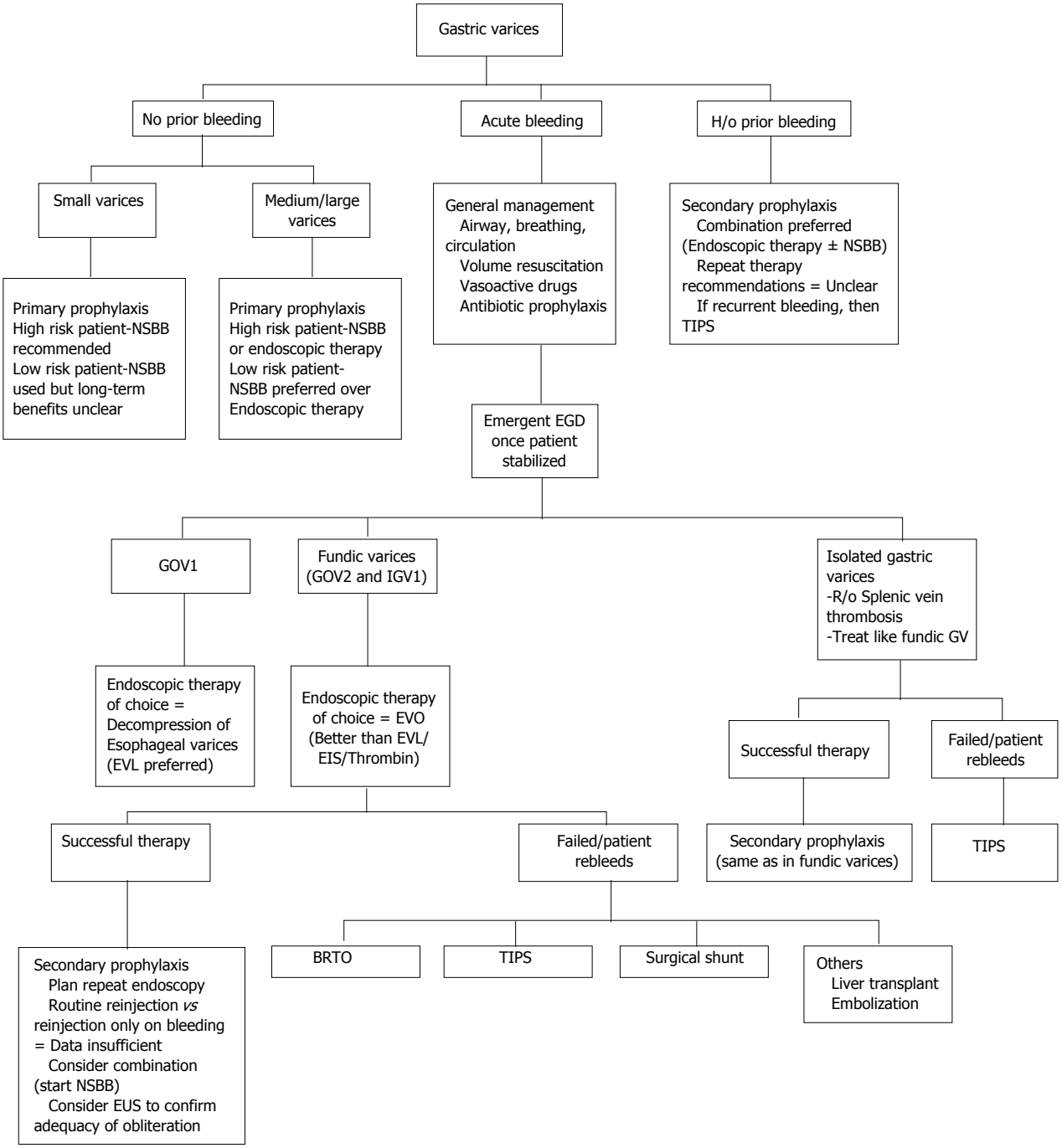

Figure 1 Proposed management algorithm for gastric varices. High risk patient: Child Pugh class B or C or endoscopic presence of red wale sign; Low Risk Patient: Child Pugh class A and no endoscopic high-risk features. GV: Gastric varices; EVO: Endoscopic variceal obliteration; EVL: Endoscopic variceal ligation; EIS: Endoscopic injection sclerotherapy; NSBB: Non specific beta blocker; BRTO: Balloon-occluded retrograde transvenous obliteration; TIPS: Trans-jugular intra-hepatic porto-systemic shunt; GOV: Gastro-oesophageal varices; IGV: Isolated gastric varices.

in management of bleeding fundic varices[1-3]. In head-to-head studies, EVL was less effective than endoscopic obturation by injection of cyanoacrylate for hemostasis of large GVs[11], and had higher re-bleeding rates too[12]. Smaller studies have attempted improvisation of ligation methods to increase its success in GV, like using detachable snares and elastic bands or in combination with sclerotherapy, however these experimental techniques are yet to be implemented universally[13,14].

Endoscopic injection sclerotherapy: Fundic varices (IGV1 and IGV2) are wider and have larger volume, needing large quantity of sclerosant which is susceptible to being washed away, potentially leading to systemic (esp. pulmonary) embolization, and may also lead to increased chances of ulceration at injection site. Before the advent of newer techniques, sclerotherapy of GVs using alcohol or tetradecyl sulfate was common and was associated with decent initial hemostasis rates (up to 67%-100%),

however the higher frequency of re-bleeding mainly due to post-procedure ulceration severely limited its long-term success[10]. Furthermore, the risk of complications including fever, retrosternal chest pain, temporary dysphagia and pleural effusions was unacceptably higher with EIS[15]. Overall, the success of EIS is questionable in management of acute GV bleeding[16] and hence is not the preferred method in any of the guidelines.

Endoscopic variceal obturation: Endoscopic variceal obturation (EVO) using tissue adhesives like glue, cyanoacrylate or histoacryl has provided a positive direction to management of fundic varices, which was always a challenge. Cyanoacrylate is a polymer which upon coming in contact with blood polymerises instantly leading to obliteration of varices. It is called "obliteration" and not "eradication" since the varices may be still visible post-treatment.

EVO with N-butyl-2-cyanoacrylate has been the advocated first-line method in managing the gastric varices especially fundic varices[1-3]. Kang *et al*[17] performed EVO with cyanoacrylate in 127 patients with GVs (100 active bleeding and 27 prophylactically) and reported a primary hemostasis rate of 98.4% (1 session-98 patients, 2 sessions-25 patients, ⩾ 3 sessions-4 patients), with a recurrent bleeding rate of 18.1 % at 1 year[17]. Several studies have compared EVO head-to-head with EIS or EVL to conclude the favorable outcomes of EVO in terms of initial hemostasis, and lesser re-bleeding and complications[11,12,18-20]. Furthermore, re-bleeding rates after EVO were found to be comparable to transjugular intrahepatic porto-systemic shunt (TIPS) in patients with acute GV bleeding, suggesting this technique may be equally efficacious in secondary prevention and creating opportunity of therapy in patients in who TIPS is contraindicated for encephalopathy reasons[21]. Few studies have advocated using dynamic CT scan prior to EVO to increase the detection of feeding vessels, assessment of direction of blood flow, presence of shunts, in an attempt to increase efficacy and minimize complications of EVO technique[22], although this is not universally practiced.

Although EVO is clearly a superior technique than EIS or EVL for bleeding GVs, it is not free of technical difficulties (para-variceal injection, needle sticking in the varix, intra-peritoneal injection leading to peritonitis and adherence of the glue to the endoscope) or complications (fever, para-variceal injection with mucosal necrosis and bleeding, embolization into the renal vein, IVC, pulmonary or systemic vessels and retro-gastric abscesses)[12,18-20]. However, emerging literature supports preference of distilled water over saline to dilute cyanoacrylate to decrease coagulation and use of standardized techniques of tissue adhesive preparation and delivery to decrease rates of these complications[23]. In case of large gastric varix, it is advised to begin tissue adhesive injection from bottom to dome to minimize risk of bleeding if injected directly at high pressure-high flow dome area. Liu *et al*[24] reported an interesting scenario which developed when EVO of GVs led to hemorrhage from EVs due to embolism of the glue into the EV thus increasing the pressure. This was not amenable to EV ligation due to presence of foreign body (glue) and was managed with cyanoacrylate injection into EVs to achieve hemostasis and authors rightly cautioned endoscopists to treat EVs in the same setting as EVO of GVs to prevent such a complication[24].

Another major difference between EVO and other endoscopic techniques is that variceal obliteration of the GVs is not quite obvious after cyanoacrylate injection, and hence adequacy of EVO is controversial. Most often GVs are probed with an endoscope and the induration is accepted as a sign of inadequate obliteration with the need to inject more tissue adhesive till it is "hard" to palpate. Improved radiology (use of CT portography)[22] and newer endoscopic techniques have made this EVO adequacy assessment easier, as discussed later in this article. Notably, EVO has recently been shown to be superior to beta blocker therapy for primary prophylaxis of GVs and hence is being advocated[25]. Evidence regarding efficacy of the glue in pregnant females and in children is still emerging and premature, and so is data on newer combination EVO-sclerotherapy modalities[26].

Novel EVO materials: Endoscopists are trying several materials to achieve hemostasis in technically challenging situations, like successful use of hemostatic powder in situation with failed EVO with cyanoacrylate glue and contraindication to TIPS due to dilated cardiomyopathy[27]. Thrombin was used by Yang *et al*[28] and Ramesh *et al*[29] in separate studies to successfully achieve initial hemostasis 100% and 92% patients, with re-bleeding rates of 27% and 0% respectively. Thrombin helps in clotting by converting fibrinogen to fibrin and promotes platelet aggregation as well. Although these studies were limited by their patient size (12 and 13 patients respectively)[28,29], and did not report any untoward thrombo-embolic events, the concern for thrombin leakage into systemic circulation and potentially causing disseminated intravascular coagulation (DIC) or systemic embolization still remains. It is currently not being advocated due to lack of adequate data.

Role of endoscopic ultrasound: It is common knowledge that endoscopic ultrasound (EUS) enables the visualization of esophago-gastric varices and other venous collaterals viz. peri- and para-esophageal collateral veins and perforating veins, in patients with PHT, and can be useful to assess the patency of the portal venous system[30]. There has been an attempt in 1993 to classify gastric varices endosonographically by Boustière *et al*[31], which considered size of GVs and gastric wall abnormalities (Table 1), and inferred that while endoscopy graded EVs better, EUS was a better tool to classify GVs and early signs of portal gastropathy. The other EUS features of portal hypertension, in addition to EVs and GVs, may include dilatation of the azygos vein, splenic vein

Table 1 Endoscopic ultrasound classification of gastric varices: Proposed by Boustiere *et al* in 1993

Endoscopic ultrasound classification of gastric varices	
1: Size of gastric varices	Grade 0 (none) Grade 1 (small or non-confluent varies < 5 mm) Grade 2 (large or confluent varices ⩾ 5 mm)
2: Abnormalities of gastric wall	Grade 0 (none) Grade 1 (thickening and brilliance of the third hyperechogenic layer with or without fine internal anechogenic structures) Grade 2 (visible vessels in the third layer which deform the entire wall, with penetrating varices)

and portal vein, increased diameter of the thoracic duct, thickening of gastric mucosa and submucosa, presence of portal hypertensive gastropathy, and the presence of rectal varices[30,32]. In addition, EUS combined with color Doppler imaging enabled visualization of shunts viz. gastro-renal shunt in one report[33]. Furthermore, EUS doppler helps characterize gastric submucosal lesions better than EGDs before proceeding to the biopsy of potential GV.

Role of EUS in risk estimation for GV bleeding is a field of growing interest. EUS probes can be used to measure size of varices (diameter), and furthermore to estimate variceal wall thickness which is deemed as a better predictor of bleeding than varices diameter alone[34]. Intra-variceal pressure measurement may be a better surrogate for risk of bleeding, which can be accomplished by direct variceal puncture which is not practiced because of invasiveness. Although data is slim, there has been an attempt looking at EUS guided EV pressure recording, to better predict risk of bleeding, and it has been shown to have reasonable correlation with hepatic venous pressure gradient (HVPG)[35]. Finally, high risk stigmata like red hematocytic spot can be visualized with EUS[36].

EUS-assisted injection sclerotherapy for both gastric[37] and esophageal varices[38] is effective, achieving high eradication and low recurrence rates in long-term follow-up. In fact the risk of re-bleeding after EUS directed sclerotherapy is reportedly lower than endoscopic technique. Recently additional attention has been diverted towards EUS delivered therapies to control bleeding in acute variceal bleeding patients, using unique agents like adhesive tissue (histoacryl)[39], thrombin[40] and EUS-guided coil injection for gastric[41] and ectopic duodenal varices[42]. Last but not the least, EUS finds its utility in confirmation of adequacy of EVO of gastric varices, eliminating the need for inept endoscope probing assessment and thus increasing overall efficacy of EVO technique[43]. A recent study from Taiwan used miniature ultrasound probe (MUP) sonography in 34 patients who underwent cyanoacrylate EVO therapy for acute GV bleeding, during follow-up endoscopy session to assess adequacy of obturation and reinjection if necessary. The authors demonstrated a significantly greater free-of-rebleeding rate and trend towards better survival for patients in MUP group compared with conventional endoscopy group[43]. Although these advances bring a sound of promise, EUS probe which has a larger diameter compared to conventional scope, in addition to GV intervention is certainly a high-risk procedure. Using a mini-probe may counter some of this added disadvantage but non-availability of pediatric sizes is still a limitation. Furthermore, future studies need to compare radial and linear EUS scopes in diagnosis and management of varices.

Indirect approach

Decreasing portal pressure - either surgically or percutaneously by establishing a TIPS.

Role of TIPS: Porto-systemic shunts such as TIPS are typically advocated as second-line acute therapy (after endoscopic management) to prevent re-bleeding of varices[1-3]. Although decreasing portal pressure is considered effective in reducing the bleeding rate of EVs, it is inconsistently effective for GVs, which tend to occur and bleed at lower portal pressures[21,44]. Also there is discordance between decreased hepato-portal gradient with TIPS and actual decrease in GV re-bleeding. In addition, TIPS has its own limitations including worsening of encephalopathy or shunt occlusion, which can lead to recurrence of hemorrhage, and surveillance for patency.

Role of advanced radiological procedures: If all endoscopic techniques and TIPS fail or if TIPS is contraindicated, then the next step would be balloon-occluded retrograde transvenous obliteration (BRTO)[1-3], which is a popular technique in Japan, and allowing modulation of flow within the varices. BRTO was popularized and named by Kanagawa *et al*[45] in 1996, this technique optimize the action of the sclerosing agent by inducing stagnation in the gastric varices, thereby allowing maximal sclerosant dwell time to cause endothelial sclerosis and vascular thrombosis. The discussion of technique, advantages and complications of BRTO is beyond the scope of current mini-review, but one of the emerging fronts in management of acute GV bleeding.

CONCLUSION

GVs are notorious to bleed massively and often difficult to manage with conventional techniques. EVO with cyanoacrylate glue injection is currently the most favored for being superior to variceal ligation or sclerotherapy in achieving hemostasis in acute gastric variceal bleeding. Endoscopists must remain cognizant about the possible complications of tissue adhesive injections and strive for standardization of EVO techniques to minimize them. Novel techniques like use of thrombin, coil embolization are under investigation as alternatives to cyanoacrylate aiming for improved outcomes. TIPS and BRTO are advanced radiological procedures available as

salvage techniques in uncontrollable bleeding situations or when patients are not candidates or have failed endoscopic management. The role of EUS in the therapeutic algorithm for GVs is still evolving. EUS is being used to confirm presence, size and location of GVs, to stratify the risk of re-bleeding, as a therapeutic tool to perform sclerotherapy or EVO, and to confirm eradication of GVs after EVO. Emerging use of EUS provides optimism of better diagnosis, improved classification, innovative management strategies and confirmatory tool for eradication of GVs.

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MINIREVIEWS

Role of intrahepatic innervation in regulating the activity of liver cells

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Abstract

Liver innervation comprises sympathetic, parasympathetic and peptidergic nerve fibers, organized as either afferent or efferent nerves with different origins and roles. Their anatomy and physiology have been studied in the past 30 years, with different results published over time. Hepatocytes are the main cell population of the liver, making up almost 80% of the total liver volume. The interaction between hepatocytes and nerve fibers is accomplished through a wealth of neurotransmitters and signaling pathways. In this short review, we have taken the task of condensing the most important data related to how the nervous system interacts with the liver and especially with the hepatocyte population, how it influences their metabolism and functions, and how different receptors and transmitters are involved in this complex process.

Key words: Liver innervations; Hepatocytes; Neurotransmitters; Cellular receptors

Core tip: Liver innervation comprises sympathetic, parasympathetic and peptidergic nerve fibers, organized as either afferent or efferent nerves with different origins and roles. Their anatomy and physiology have been studied in the past 30 years, with different results published over time. Hepatocytes are the main cell population of the liver, making up almost 80% of the total liver volume. The interaction between hepatocytes and nerve fibers is accomplished through a wealth of neurotransmitters and signaling pathways.

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INTRODUCTION

Innervation of the liver comprises efferent and afferent nerves containing sympathetic, parasympathetic and peptidergic fibers. Sympathetic nerve fibers derive from splanchnic nerves and the parasympathetic counterparts have a vagal origin. Fibers derived from splanchnic, vagus and sometimes the phrenic nerves enter the liver through the hilum, together with the hepatic artery, portal vein and bile duct. Some nerve fibers do not accompany hepatic vessels and enter the liver *via* the small omentum or the hepatic vein. Sympathetic and parasympathetic nerves form two separate plexus but communicate with each other: the anterior plexus placed around the hepatic artery, consisting of nerve fibers with their origin in the celiac ganglion and posterior vagus nerve, and the posterior plexus located around the portal vein and bile duct,

formed by fibers from the celiac ganglion and the right vagus[1]. Nervous fibers which are distributed to the hepatic parenchyma derive from a corresponding nervous plexus and their intrahepatic distribution differ according to species[2,3].

In the human liver, nerve endings are located in the hepatic lobules[4], which consists of hepatocytes and non-parenchymal cells. Unlike hepatocytes, which occupy almost 80% of liver volume and have numerous functions, non-parenchymal liver cells occupy only 6.5% of the liver, although representing 40% of total liver cells[5].

Hepatocytes are arranged as cellular cords with a radial disposition that converges towards the centrilobular vein, being separated by sinusoidal capillaries. Between hepatocyte cell cords and sinusoid capillaries there is an interstitial space, a perisinusoidal called a Disse space. This space is formed by a fine network of reticulin fibers, a support for the sinusoids, non myelinated nerve fibers and mesenchymal type cells[6]. Non-parenchymal cells are located in the liver sinusoidal compartment. The hepatic sinusoidal wall consists of three cell types: sinusoidal endothelial cells (SECs), Kupffer cells (KCs) and hepatic stellate cells (HSCs)[5]. Most nerve endings from intralobular spaces are located in Disse spaces[4,7-12], where they make close contact with HSCs, SECs and hepatocytes[7,8,10].

NERVOUS INFLUX TRANSMISSION MECHANISM INTO HEPATOCYTES

Hepatocytes serve multiple functions, such as synthesis, storage, metabolism and transformation of carbohydrates, amino acids, proteins, lipids, vitamins and detoxification, conjugation and excretion of exo- and endogenous substances. During liver regeneration, hepatocytes initiate cell proliferation, maintain metabolic function of the liver, secrete interleukin-6 (IL-6), proteases, protease inhibitors and hepatocyte growth factor[13].

The liver receives both sympathetic and parasympathetic nerve fibers; however, the innervation that hepatocytes receive varies by species. Thus, in the cat, rabbit, guinea pig liver as well as primate liver, it appears that nerve endings are connected to all hepatocytes, unlike rats and mice in which only hepatic cells in the portal region appear to be in contact with intrahepatic nerve endings[14].

Nerve fiber communication with hepatocytes can be accomplished by several mechanisms (Figure 1): (1) hepatocyte direct innervation mediated by norepinephrine and acetylcholine, neuropeptides [neuropeptide Y, galanine (NPY), vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), *etc.*], purines [adenosine triphosphate or adenosine (ATP)]; (2) signals intercellular transmission using gap type junctions; and (3) sinusoidal capillary cells innervation which interact with hepatocytes through eicosanoids (prostaglandins, leukotrienes), cytokines (necrotic factor, IL-6, IL-1) and other chemical mediators (endothelin, nitric oxide).

Direct innervation of hepatocytes

Nerve transmission to hepatocytes is achieved through neurotransmitters such as norepinephrine and acetylcholine, neuropeptides, such as NPY, galanine, VIP and CGRP, or purine derivatives as ATP and adenosine.

The liver is stimulated by norepinephrine and epinephrine released from intrahepatic nerve endings but also derived through the blood from the adrenal glands. Catecholamines act in the liver on α1-, α2- and β2-adrenergic receptors[15-17]. Norepinephrine is removed from the site of action by intrahepatic nerve ending uptake, being degraded by liver cells and diffused through the vascular bed[1].

Experiments on rat liver have shown that stimulation of the autonomic nerve plexus around the hepatic artery and portal vein causes increased production of glucose and lactate[1], urate and allantoin formation[17], decreased ketogenesis[18], increased ureogenesis and ammonia uptake[19], as well as increased oxygen utilization[20,21]. Also, hepatic nerve stimulation leads to decreased[16,17,20,22,23] and redistributed intrahepatic flow[21], as well as raised noradrenaline levels in the hepatic vein[15-18]. All these effects of hepatic nerves are only possible in the presence of extracellular calcium[22,24].

NPY, galanin, SP, CGRP, VIP and purine derivatives (ATP, adenosine) act as neurotransmitters, both in adrenergic and cholinergic nerve fibers, as well as in the related hepatic nerves. These neurotransmitters are released locally and are involved in regulating hepatic microcirculation. NPY and ATP act as vasoconstrictors, while VIP, CGRP, SP and adenosine produce vasodilation[14].

Some neurotransmitters also have a metabolic function. Thus, sympathetic hepatic nerve stimulation causes the release of noradrenaline but also of galanin[25], suggesting that galanin potentiates the action of norepinephrine to stimulate hepatic glucose production under stress[14].

Yamamoto *et al*[26] revealed the metabolic activity of ATP, which potentiates the action of hepatic sympathetic nerves suppression action on the formation of ketone bodies in the liver, the effect probably being due to ATP interaction with norepinephrine[14].

Intercellular transmission of signals through gap type junctions

Intrahepatic innervation varies by species. In some species, most hepatocytes are not directly innervated but there is an indirect mechanism for transmitting nervous inflow. One such mechanism is the intercellular communication carried out between adjacent hepatocytes *via* specific channels known as gap type junctions (GJ), which allow the passage of ions and small molecules[14].

GJ density is different among species[27]. Thus, hepatic GJ are more numerous in rats and mice compared to rabbits and guinea pigs[14].

GJ are membrane channels that allow intercellular communication between neighboring cells. GJ consists of two hemichannels, one hemichannel belonging to each

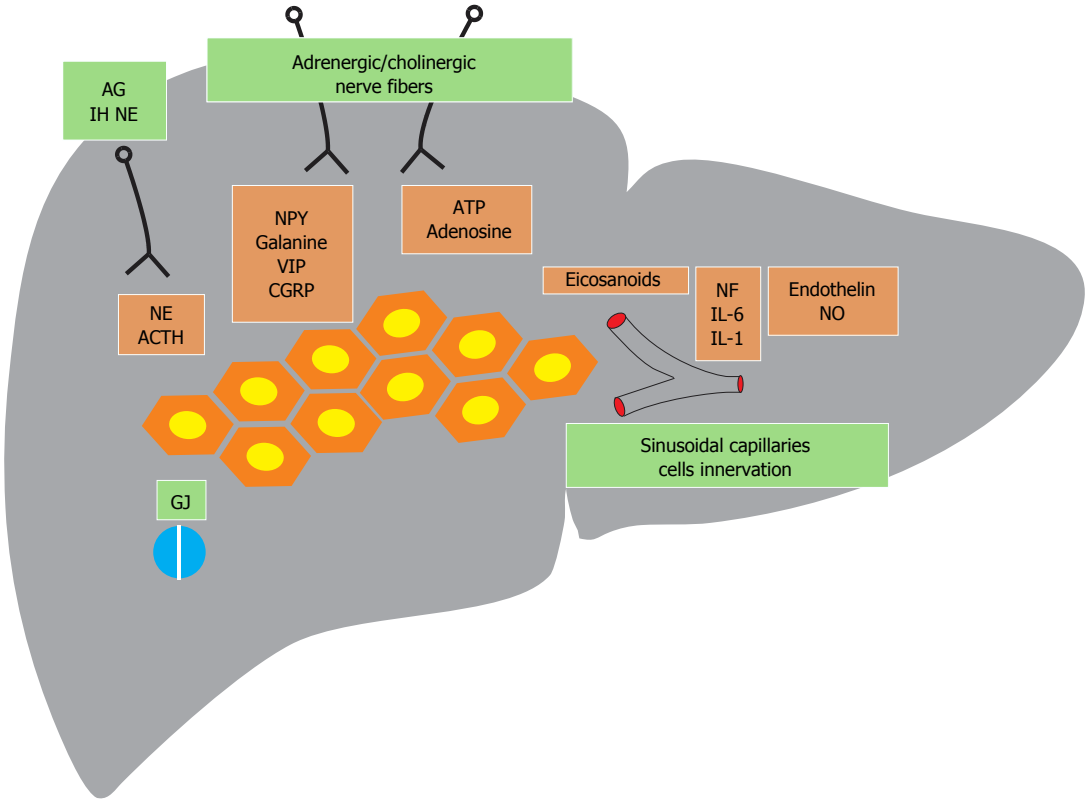

Figure 1 Schematic representation of the communication pathways between nerve fibers and hepatocytes. AG: Adrenal gland; IH NE: Intrahepatic nerve endings; ATP: Adenosine triphosphate; NPY: Neuropeptide Y; VIP: Vasoactive intestinal peptide; CGRP: Calcitonin gene-related peptide; IL: Interleukin; NF: Necrotic factor; NO: Nitric oxide; GJ: Gap junction.

of the two adjacent cells. A hemichannel consists of six subunits or connexins. Connexin 32 (Cx32) is the major protein component expressed in murine hepatocytes. Cx32 plays an essential role in signal propagation induced by the norepinephrine released from sympathetic nerve endings in hepatocytes[28].

GJ ensure the transmission of information to neighboring cells, achieving functional integration of hepatocytes, and thus functioning as a body and not as a mere cluster of cells[28].

GJ have an important role in transmitting nervous impulses from sympathetic nerve endings in parenchymal and non-parenchymal cells of the liver, in some species of mammals. There is an inverse relationship between sympathetic nerve fiber density and number of intrahepatic GJ[14]. Research on rat liver, which contains numerous GJ[15,29], showed that sympathetic nerves innervate only a part of parenchymal and non-parenchymal cells of the periportal area[15]. Metabolic and hemodynamic effects of hepatic sympathetic nerves are achieved by α1 receptor stimulation[17,23,30], are abolished by prostanoids synthesis inhibitors, and are mimicked by prostaglandins but not by thromboxanes[31]. Norepinephrine, as well as F2α prostaglandin, stimulates the release of glucose from isolated hepatocytes with increased inositol 1,4,5-triphosphate and glycogen formation[32].

Sending signals through GJ is involved in the metabolic effects of sympathetic nerves[14]. The norepinephrine released from sympathetic nerve endings binds to α1 receptors of parenchymal and non-parenchymal cells. The release of glucose from the proximal parenchymal cells from the periportal region under the action of norepinephrine initiates a signal which propagates through GJ to distal parenchymal cells, which in turn releases glucose. Norepinephrine stimulates contraction of proximal non-parenchymal cells (sphincters), reducing the flow in the sinusoid capillaries. It also causes the release of prostaglandins (PG) into the Disse space. PG, in turn, bind to prostanoid receptors of parenchymal and non-parenchymal cells. PG released from non-parenchymal cells are rapidly degraded in the liver[32] and so do not reach the distal cells. PG increase glucose release in the periportal parenchymal cells and perhaps initiate a signal that propagates to the other cells by GJ. PG stimulate contraction of proximal non-parenchymal cells, thus reducing the flow to sinusoid capillaries[15,33].

Sinusoidal capillary cells innervation

Sinusoidal capillaries are located between the cell cords and have a 9 to 12 micrometers diameter. The sinusoidal capillary wall is discontinuous and is formed out of a basement membrane and a capillary endothelium. Sinusoidal endothelium consists of flattened endothelial cells and phagocytic Kupffer cells in a ratio of 5/3. In addition to these two main types of cells in the sinusoid capillaries, there are also stellate cells and lymphocytes[6]. Capillary walls have an important role in the regulation

of the sinusoidal microcirculation[34,35].

Endothelial cells are flat and have elongated, hyperchromic nuclei and reduced cytoplasm. Junctional complexes are lacking. There are very small spaces of 0.5 micrometers between the cells. The apical membrane of the endothelial cells has transcytoplasmic fenestrations which are small holes arranged in nests with a diameter of 100 nm, with a role in controlling cholesterol, lipoprotein and vitamin A metabolism. In the cytoplasm of endothelial cells, a small number of cell organelles and pinocytosis vesicles were revealed. Endothelial cells produce prostaglandins, endothelin, IL-1 and IL-2[6,13].

Hepatic stellate cells (Ito cells, lipocytes) are located in the Disse space in small niches, among hepatocytes. They are in contact with the liver cells and through their microvilli and cytoplasmic processes have contacts with endothelial cell microvilli[6]. Well developed organelles and lipid droplets were found in the HSC cytoplasm. HSCs store vitamin A, containing factors related to retinoid acid and retinol, and produce extracellular matrix. Under normal conditions, HSCs have a deposit function, while in pathological ones it transforms into myofibroblastic type cells[6,13,36,37].

Research has shown that the distribution of intrahepatic innervation varies by species[29,38-43]. Guinea pig, cat and tupaia have an intralobular innervation similar to the human one[11,12,29,40], in contrast to mice and rats where it differs from the human[39,41-43].

In human liver, nerve endings are located in the Disse space[4,11,12], closely connected to hepatocytes and non-parenchymal cells, particularly HSCs[7,8,10,11].

Non-parenchymal cells are the only ones that can synthesize eicosanoids (prostaglandins, thromboxanes and leukotrienes) from arachidonic acid released from phospholipids by the action of phospholipase A_2 and converted to prostaglandins and thromboxanes *via* the cyclooxygenase path and leukotrienes *via* the lipoxygenase path[1]. Experiments on perfused rat liver have shown that the synthesis and secretion of prostanoids in non-parenchymal liver cells is influenced by a number of physiological stimuli, pathological and chemical. These stimuli also determine an increased release of glucose and lactate, as well as increased vascular resistance in the liver[1]. Of these stimuli, the most important are: extracellular nucleotides[44], nucleosides[45], zymosan[46,47], endotoxins[48], aggregates of immunoglobulins[49], anaphylatoxins[50,51], phorbol esters and calcium ionophores[44]. Norepinephrine and/or other chemical mediators released from nerve endings can stimulate the formation of prostanoids in non-parenchymal liver cells. Prostanoids, in turn, can modulate hepatocyte metabolism[1].

Of eicosanoids, only PG, without thromboxanes and leukotrienes, play a role in the events triggered by nerve stimulation[1].

PG participation in the chain of events initiated by nerve stimuli in the liver depends on hepatocellular receptors for PG. Research conducted so far confirms the existence of these receptors[1].

HSCs are indirectly involved in nerve fiber communication with hepatocytes, through PG. Noradrenaline may lead, by means of α1-adrenergic receptors, to increased synthesis of PG in the HSC, and PG, in turn, stimulate glycogenolysis in the hepatocytes. Unlike KC, producing predominantly PGD_2, HSC secret PGF 2 alpha released in increased amounts compared with PGD_2, as a result of sympathetic stimulation[52].

Intrahepatic nerve fiber terminations, often containing vesicles which contain neurotransmitters like substance P (SP) and vasoactive intestinal peptide (VIP), are closely related to HSC. It is considered that HSC that surround CECS, forming sinusoidal capillary walls, have a role in the contraction and relaxation of sinusoidal walls, thus intervening in the regulation of the sinusoidal microcirculation[4].

HSC contraction is stimulated by a number of substances such as endothelin-1 (ET-1), angiotensin II, norepinephrine, prostaglandin F_2, thromboxane A_2 and thrombin. In contrast, vasoactive substances such as acetylcholine, VIP, nitric oxide (NO), carbon monoxide, prostaglandin E_2 and adrenomedulin produce HSC relaxation[4].

ET-1 produces contraction of HSCs through ET receptor stimulation on autocrine or paracrine pathways. HSC contraction appears to be related to the increase of intracellular Ca^{2+} and inositol phosphate. For the sinusoid microcirculation control role of the HSC, the presence in the HSC cytoplasm of α smooth muscle actin, which is a contractile protein, also stands, such that the contraction of the HSC can be compared with that of smooth muscle cells in the vessel wall structure. On the other hand, prostaglandin E_2, adrenomedulin and other vasoactive substances determine HSC relaxation by increasing intracellular cAMP. In addition, HSC produces NO and inhibits contractility by an autocrine mechanism linked to NO[8].

Of the mentioned vasoactive substances, ET-1 and NO have an important role in the regulation of sinusoidal microcirculation. ET is a peptide consisting of 21 amino acids with a strong vasoconstrictive effect on the smooth muscle fibers. It has three isoforms, ET-1, ET-2 and ET-3[4,53].

Two receptors have been identified for ET: ET_A and ET_B, both belonging to the superfamily of G-protein-coupled receptors[54]. ET_A receptor has a higher affinity for ET-1 and ET-2 than for ET-3, while the ET_B receptor has a similar affinity for all three isoforms of ET. ET_A receptors stimulation increases intracellular cAMP levels, whereas ET_B receptor stimulation leads to inhibition of the adenylate cyclase system[55]. Also, ET_B receptor stimulation activates Ca^{2+}-dependent NOS[56]. Douglas *et al*[57] described two ET_B receptor subtypes: ET_{B1} and ET_{B2}. Stimulation of ET_A and ET_{B1} receptors causes contraction of smooth muscle fibers, while ET_{B2} receptor stimulation causes dilation by increased synthesis of NO[58].

ET-1 receptors from intralobular spaces predominate in the juxtaportal region. About 35% of ET-1 receptors

are located in the HSCs, a smaller number are located in the CECS and KCs[59]. Both ET_A and ET_B receptors are found in the liver. All cells of the sinusoidal capillary walls have ET_B receptors but only HSCs have ET_A receptors. Mallat *et al*[60] have identified 20% ET_A receptors and 80% ET_B receptors on activated HSCs.

NO is synthesized from L-arginine by the NO synthetase path (NOS). Three NOS isoforms have been identified: two are calcium-dependent, one produced by the neurons (nNOS or NOS I) and other by the vascular endothelial cells (eNOS or NOS Ⅲ), and one calcium-independent isoform, cytokine-induced (iNOS or NOS II). Rat HSCs shrink under ET-1 or SP action and relax, causing vasodilatation, under NO action produced by HSC under the influence of IL-1[4,61].

In normal liver, sinusoidal contraction is inhibited by ET_A receptor antagonists[62,63] but, according to some authors, not by ET_B receptors antagonists[4,62-64]. Other researchers believe, however, that ET_B receptor stimulation results in constriction of sinusoid capillaries[65]. It is possible that this discrepancy between the results obtained by different authors is due to coupling ET_B receptors with NOS, which masks the vasoconstrictor effect[63,66].

The various subtypes of endothelin have different effects on hepatic microcirculation. The relationship between NO and endothelin is extremely important in the control of vascular tone[63].

Liu *et al*[58] have shown that ET-1 binding to ET_B receptor leads to eNOS activation on the Akt phosphorylation path, thus reducing the phosphorylation of eNOS and NO synthesis. The same researchers highlighted the crucial role of βγ subunits of the G protein in triggering endothelin/NO reactions. The stimuli which regulate the ET expression and vascular sensitivity to ET also adjust the NOS and heme oxygenase-1 activity. Both enzymes catalyze the production of substances which, by guanylate cyclase activation, produce vasodilation[67].

CONCLUSION

Liver innervation is one of the most complex control systems in the human body; therefore, a better understanding of its inner workings is of paramount importance for developing future therapies and procedures for ameliorating the metabolic function of the liver. Being able to manipulate nerve impulses and synaptic mediators can possibly allow direct control over the functions of hepatocytes. Direct acting agents with excellent control over specific liver functions could become a reality, with direct implications for drug therapy, surgery or liver transplant.

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MINIREVIEWS

Hepatoprotective effect of silymarin

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Abstract

The use of medicinal plants in treating illnesses has been reported since ancestral times. In the case of hepatic diseases, several species such as *Silybum marianum*, Phyllanthus niruri, and *Panus giganteus* (Berk.) have been shown to ameliorate hepatic lesions. Silymarin is a natural compound derived from the species *Silybum marianum*, which is commonly known as Milk thistle. This plant contains at least seven flavoligands and the flavonoid taxifolin. The hepatoprotective and antioxidant activity of silymarin is caused by its ability to inhibit the free radicals that are produced from the metabolism of toxic substances such as ethanol, acetaminophen, and carbon tetrachloride. The generation of free radicals is known to damage cellular membranes and cause lipoperoxidation. Silymarin enhances hepatic glutathione and may contribute to the antioxidant defense of the liver. It has also been shown that silymarin increases protein synthesis in hepatocytes by stimulating RNA polymerase I activity. A previous study on humans reported that silymarin treatment caused a slight increase in the survival of patients with cirrhotic alcoholism compared with untreated controls.

Key words: *Silybum marianum*; Hepatoprotector; Lipoperoxidation; Silymarin

Core tip: One of the mechanisms of liver damage caused by alcohol is the generation of free radicals formed by the metabolism of this xenobiotic. Silymarin is an antioxidant that protects the liver from the free radical damage produced by alcohol metabolism. Silymarin is the most used natural compound for the treatment of hepatic diseases worldwide due to its antioxidant, anti-inflammatory, and anti-fibrotic activities. Silymarin functions by stabilizing biological membranes and increasing protein synthesis.

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INTRODUCTION

The liver is an important organ that has a key role in the

maintenance of homeostasis. The liver is responsible for multiple metabolic functions and physiological processes such as bile production, energy generation, vitamin storage, and the metabolism of carbohydrates, proteins, and lipids. After intestinal absorption is complete the blood is rich in nutrients and xenobiotics. The blood is then transported to the liver *via* the portal vein, which carries multiple toxic substances including ethanol (Et-OH), drugs, pharmaceuticals, and toxins to the liver. As a result, the liver is susceptible to toxicity and damage. Many people have been afflicted with some type of liver lesion. Examples of liver lesions include fatty liver, non-alcoholic steatosis, hepatitis A, B, or C, cirrhosis, and hepatocellular carcinoma (the third leading cause of cancer-related mortality worldwide)[1]. Hepatic diseases are primary causes of morbidity and mortality worldwide. The most recent surveillance report published by the National Institute on Alcohol Abuse and Alcoholism showed that liver cirrhosis was the 12th leading cause of death in the United States[2]. Liver disease is exacerbated by unhealthy lifestyles, obesity, and the excessive consumption of alcohol and drugs[3].

The use of medicinal plants has been reported since ancestral times. In the case of hepatic diseases, several species such as *Silybum marianum*[4], *Phyllanthus niruri*[5], and *Panus giganteus* (Berk.) have been shown to ameliorate hepatic lesions[6].

SILYMARIN

Overview

Flavonoids are polyphenol compounds that are also considered essential nutrients. Their basic chemical structure consists of two benzene rings bound by a three-atom heterocyclic carbon chain. The oxidation of the structure generates several families of flavonoids (flavones, flavonoles, flavanones, anthocyanins, flavanoles, and isoflavones). Chemical modifications of each family can lead to > 5000 individual compounds with different properties[7].

Silybum marianum is the scientific name for Milk thistle or St. Mary's thistle. It is a plant native to the Mediterranean region and belongs to the Asteraceae family. It is characterized by thorny branches and a milky sap, with its oval leaves reaching up to 30 cm. The flowers are bright pink and can measure up to 8 cm in diameter[8]. Milk thistle grows in its wild form in southern Europe, northern Africa, and the Middle East. The plant is cultivated in Hungary, China, and South American countries such as Argentina, Venezuela, and Ecuador. In Mexico, Milk thistle is consumed as a supplementary food[9].

Silymarin is a natural compound that is present in species derived from *Silybum marianum*, which is commonly known as Milk thistle. The plant contains at least seven flavolignans and the flavonoid taxifolin. The most important flavolignans present include silybin, silydianin, and silychristine. Silybin represents between 50% and 70% of the extract from silymarin. The following flavolignan isoforms are known (Figure 1): silybin A, silybin B, isosilybin A, and isosilybin B[10]. Silymarin has been used worldwide for many years as a complementary alternative medicine because of the beneficial effects associated with the treatment of hepatic diseases. Silymarin belongs to the Aster family (Asteraceae or Compositae). The mature plant has large brilliant-purple flowers and abundant thorns. The plant grows in places with sufficient sun exposure[11].

The low level of bioavailable flavolignans is known. For example, the level of silymarin absorption is between 20% and 50%. Silybin is the major compound of silymarin and limiting factors such as low solubility in water, low bioavailability, and poor intestinal absorption reduce its efficacy. New soluble silybin-derived biocompounds (silybin bis-hemisuccinate, β-cyclodextrin complex, silybin-*N*-methyl-glucamine, silybin 11-*O*-phosphate, and silybin-phosphatidylcholine) have thus been designed[10]. Chronic inflammation occurs in patients with hepatic damage. Thus, for patients with compensatory cirrhosis, hepatitis C, and non-alcoholic hepatic steatosis, the bioavailability of compounds present in silymarin may be affected, which may also explain the low effectiveness of treatment with flavonoids in these patients[12,13].

Sy-Cordero *et al*[14] isolated four key flavolignans and diastereoisomers (silybin A, silybin B, isosilybin A, and isosilybin B) from *S. marianum* on a gram-scale. These compounds and two other related analogues are present in extremely minute quantities. The compounds were evaluated for their antiproliferative/cytotoxic activity against human prostate cancer cell lines. Silymarin reduces the incidence of certain cancers[15]. Su *et al*[16] used silymarin on nasopharyngeal carcinoma cells (NPC-TW01) and found an increase in Bcl-2 expression and a decrease in the activated caspase-3 or apoptosis-inducing factor (AIF) with low-dose (80 μmol/L) treatment.

The molecular targets of silymarin for cancer prevention have been studied. Milk thistle interferes with the expression of the cell cycle regulators and proteins involved in apoptosis. Thus, it can modulate the balance between cell survival and apoptosis. Lee *et al*[17] reported that silybin inhibited the kinase activity of mitogen-activated protein kinase (MEK)-1/2 and ribosomal S6 kinase (RSK)-2 in melanoma cells. The treatment of melanoma cells with silybin attenuated the phosphorylation of extracellular signal-regulated kinase (ERK)-1/2 and RSK2, which is regulated by the upstream kinases MEK1/2. The blockade of MEK1/2-ERK1/2-RSK2 signaling by silybin resulted in the reduced activation of nuclear factor-kappa B (NF-κB), activator protein-1, and STAT3. These proteins are transcriptional regulators of several proliferative genes in melanomas. Silybin blocks the activation of these transcription factors and induces cell-cycle arrest at the G_1 phase, which inhibits melanoma cell growth *in vitro* and *in vivo*. Silymarin suppresses ultraviolet radiation A-induced oxidative stress (OS), which can induce skin damage. Thus, the topical application of silymarin can be a useful strategy for protecting against skin cancer[18].

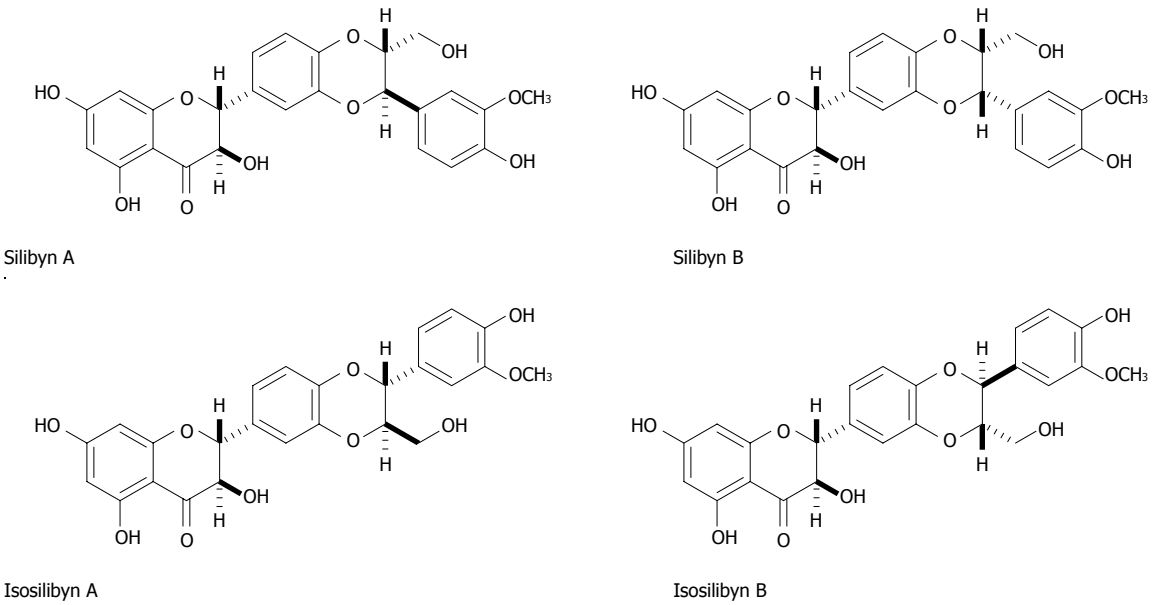

Figure 1 The chemical structure of silybin A, silybin B, isosilybin A, and isosilybin B, the extract of silymarin.

In previous studies, the inherent hepatoprotective and antioxidant activity of silymarin was shown to be caused by its control of free radicals (FR), which are produced by the hepatic metabolism of toxic substances such as Et-OH, acetaminophen (Paracetamol), or carbon tetrachloride. The FR damage cellular membranes and cause lipoperoxidation (LPO)[19]. The cytoprotective effect in liver is also caused by the inhibition of the cyclooxygenase cycle, leukotrienes, and the production of FR in Kupffer cells in mice. These affects reduce inflammation[20], and it has been suggested that silymarin also performs the following functions: protecting against genomic injury, increasing hepatocyte protein synthesis, decreasing the activity of tumor promoters, stabilizing mast cells, chelating iron, and slowing calcium metabolism, among other activities that have been described in the literature[21].

Silymarin has been reported to have antioxidant, immunomodulatory, anti-fibrotic, anti-proliferative, and antiviral properties. It also affects the synthesis of RNA and DNA. Furthermore, silymarin maintains the integrity of the hepatocyte membrane and impedes the entrance of toxic substances or xenobiotics. Due to its phenolic nature, it is capable of donating electrons to stabilize FR and reactive oxygen species (ROS). Silymarin also affects intracellular glutathione, which prevents lipoperoxidation of membranes[22].

Pure compounds extracted from silymarin have been examined in cell lines infected with the hepatitis C virus (HCV). Polyak *et al*[23] showed that silymarin inhibits the replication of an infectious HCV genotype 2a strain (JFH1) in hepatoma cell cultures. The most effective compounds were isosilbin A, taxifolin, and silybinin, and these compounds reduced virus infection. The OS level induced by HCV, the tumor necrosis factor (TNF)-α level, and the transcription factor NF-κB were affected by silbyn A and silbyn B treatment. In general, all of the compounds showed antiviral activity and reduced the OS level caused by HCV infection[24].

The use of a silymarin extract in 72 patients with non-alcoholic hepatic steatosis (non-alcoholic fatty liver disease, NAFLD) on a controlled diet led to significantly reduced levels of alanine aminotransferase (ALT) and aspartame aminotransferase (AST) (AST/ALT < 1). Another parameter evaluated was γ-glutamyl transpeptidase (γ-GT). In NAFLD patients, γ-GT is high because of obesity, hyperinsulinemia, inflammation, and changes in the membrane permeability of the hepatocytes. The level of γ-GT decreased due to the silymarin-mediated inhibition of toxins entering the cells. Additionally, silymarin permits the stabilization of hepatocyte membranes. It also reduced the level of TNF-α, which reduces inflammation. A favorable change in the hepatorenal clearance index was also observed, which suggests a reduction in the accumulation of lipids in the liver. All of these results were visible after 6 mo of treatment[4].

EVIDENCE FROM STUDIES OF SILYMARIN AS A HEPATIC PROTECTOR AGAINST ETHANOL

Silymarin has both hepatoprotective and regenerative actions. The mechanism of action is a reduction of the FR formed by toxins that damage the cell membranes (LPO) and competitive inhibition through hepatocyte external cell membrane modification. Silymarin forms a complex that impedes the entrance of toxins into the interior of liver cells. Additionally, silymarin metabolically stimulates hepatic cells and activates the RNA iosynthesis of ribosomes to stimulate protein formation[25-27]. In a study published by Sandoval *et al*[28], the authors observed a

silymarin protection effect in rat hepatic cells when they used it as a comparison factor to measure liver weight/animal weight % (hepatomegaly). The hepatomegaly was reduced compared to other groups that were administered antioxidant substances. There was no significant difference observed between the silymarin group and the silymarin-alcohol group. This result suggests liver protection by silymarin. Silymarin enhances hepatic glutathione generation by elevating cysteine availability and inducing cysteine synthesis while inhibiting its catabolism to taurine. The regulation of cysteine synthesis may subsequently contribute to the antioxidant defense[29]. Silymarin reduced collagen accumulation by 30% in biliary fibrosis induced in rats[30]. A study in humans reported a slight increase in the survival of patients with cirrhotic alcoholism compared with untreated controls[31]. Silymarin is perhaps the most frequently used natural compound for the treatment of hepatic diseases worldwide due to its antioxidant, anti-inflammatory, and anti-fibrotic activities[32].

Study conducted with guinea pigs (*Cavia porcellus*) examining hepatic fibrosis induced through the administration of Et-OH (4/kg of weight/d) for 90 d revealed a significant reduction of lesion markers such as ALT, AST, and γ-glutamyl after silymarin treatment. The gene expressions of cytochrome 450 2E1 (CYP2E1), TNF-α, transforming growth factor beta-1 (TGF-β1), and nuclear factor kappa-light-chain-enhancer of activated B cells-1 were also reduced. There was also a reduction in FR and reduced markers of fibrosis such as alpha smooth muscle actin, collagen α 1(I), and in the caspase cytotoxicity marker. However, silymarin was less effective than vitamin C in this study. This result indicates that vitamin C is more effective in reducing the markers of damage and the production of ROS during Et-OH-induced lesions[33]. Another study evaluated the hepatoprotective effect by measuring the level of antioxidants and the effect of body weight (*bw*) in rats exposed to Et-OH (1.6 g/kg of *bw* for 4 wk). The results revealed that intoxication by Et-OH influences the *bw* of rats and the levels of thiobarbituric acid reactive substances (TBARS). The activity of the enzymes superoxide dismutase (SOD) and glutathione-*S*-transferase (GST) increased significantly. Conversely, glutathione (GSH), the activity of glutathione reductase (GR), glutathione peroxidase, and catalase (CAT) were reduced by exposure to Et-OH. The rats that received silybin and ascorbic acid had attenuated lesion markers, although the effect was greater in the group that received ascorbic acid than in the group treated with silybin. The study also concluded that stopping alcohol intake favors hepatic regeneration. Thus, it is more effective to take preventive measures than to implement curative treatment[34]. A mouse study examining the antioxidant, immunomodulatory activity and vascular function of mice showed a significant increase in OS levels in animals that received ethanol (1.6 g/kg per *bw*/d during 12 wk). Ethanol increased the production of TBARS, nitrite levels, and the activity of GST. Ethanol also significantly diminished the content of GSH and the activity of SOD, CAT, GPX, and GR. Mice that received Et-OH plus silymarin (250 mg/kg of *bw*/d for 12 wk) normalized the altered parameters. In addition, the silymarin-treated mice had reduced levels of interleukin-10 (IL-10), TNF-α, interferon (IFN), IFN-γ, vascular endothelial growth factor-A, and TGF-1β. The treatment also reduced the levels of IL-4 in the blood. The results of silymarin treatment were similar to mice that received vitamin C treatment[35].

The use of Sylubim β-cyclodextrin has been studied in non-insulin-dependent patients with diabetes and alcoholic hepatopathy. Treatment with a 135-mg/d dose did not influence insulin secretion but did significantly reduce the glucose ($P < 0.03$) and serum levels of triglycerides ($P < 0.01$) compared with the placebo. These results suggest that this treatment improves the response to insulin[36].

Clinical study conducted in 170 cirrhosis patients treated with 140 mg of silymarin three times daily for 41 months showed significant improvement, especially in the subgroups with alcoholic cirrhosis and initial "Child A" hepatic disease[31]. However, the results are controversial. A meta-analysis of 13 randomized clinical assays evaluated the beneficial or detrimental effects of Milk thistle and included patients with alcoholic and/or hepatitis B and C hepatic disease. The authors concluded that according to the data, Milk thistle did not significantly influence the improvement of these diseases. Conversely, it may have negatively affected the pathological condition[37].

CONCLUSION

There is substantial evidence suggesting that silymarin treatment improves hepatic diseases. However, some of the data are contradictory. Therefore, additional molecular studies investigating the mechanisms of action for these compounds are needed. It is known that silymarin does not possess adverse effects at high doses. Thus, it is a natural compound that is widely utilized in traditional medicine and has been investigated in formal scientific studies. Diverse hepatic damage models and ethanol injury have been utilized to study silymarin because ethanol is responsible for many cases of liver damage worldwide. The current data demonstrate that the use of silymarin treatment in alcoholic cirrhosis patients may attenuate the damage. However, silymarin treatment does not affect mortality.

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BRIEF ARTICLE

Khat (Catha Edulis) as a possible cause of autoimmune hepatitis

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Abstract

AIM: To investigate the potential role of khat in triggering auto immune hepatitis.

METHODS: Patients with a history of khat use and acute hepatitis were identified using the computer database in the hepatology department at the Royal Hallamshire Hospital. They were then assessed for probability of having autoimmune hepatitis using the revised autoimmune hepatitis scoring criteria.

RESULTS: Six patients were identified. All of them had presented with acute hepatitis on a background of khat. All were male and five of these patients were of Somali origin, while one patient was from Yemen. The patients were given points on the modified autoimmune hepatitis score which is based on their liver enzymes, autoimmune screen, exclusion of viral hepatitis alcohol and drugs, immunoglobulin levels and liver histology. The patients were given a score of -4 for khat use due to its potential to cause drug induced liver injury. Five of these patients scored between 10 and 15 points, placing them in the probable group for having autoimmune hepatitis. All of these patients were treated with prednisolone and demonstrated a good response to immunosuppression.

CONCLUSION: One possibile cause of hepatotoxicity with khat could be *via* triggering of autoimmune hepatitis in a genetically susceptible individual. Further studies are needed for confirmation.

Key words: Khat; Autoimmune hepatitis; Drug induced liver injury; Acute hepatitis; Herbs

Core tip: Khat causes hepatotoxicity. One possible mechanism could be by inducing autoimmune hepatitis.

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INTRODUCTION

Khat (Catha Edulis Celestrasae) is an evergreen shrub native to East Africa and Southern Arabia. It is chewed daily by over 20 million people in these countries. Chewing khat is a popular social habit, particularly in young males, that has spread to Yemeni, Somali and East African communities living in the United Kingdom and United States[1]. The use of khat as a stimulant is increasing primarily due to immigration. The Somali population in the United Kingdom is estimated to be as high as 90000, and

concerns over health and social problems associated with chewing khat have grown due to its potential side effects including hypertension, coronary vasospasm, myocardial infarction, delayed intestinal absorption, and mood disorders, which may result from its sympathomimetic action[2].

Various studies and case reports have suggested that khat is also hepatotoxic, leading to deranged liver enzymes and also histopathological evidence of acute hepatocellular degeneration[3-7]. Recent studies in Somali populations have shown that khat can cause acute severe liver injury in humans due to its hepatotoxic effects[8,9]. Certain drugs that are known to be hepatotoxic cause liver damage by inducing an immunological response leading to a clinical presentation similar to autoimmune hepatitis (AIH). D'Souza *et al*[10] have described atypical presentation of AIH in young Somali men, although any history of khat use was not reported. Our aim was to assess the possible relationship of khat and autoimmune hepatitis in patients presenting with acute hepatitis on a background of khat use.

MATERIALS AND METHODS

The Hepatology database at Sheffield Hospitals was searched for patients referred to the Hepatology department between 2005 and 2010 with liver problems and a history of khat use. All of the patients were tested for hepatitis A, B and C serology, autoimmune profile (including antinuclear antibodies, smooth muscle antibodies and LKM-1 antibodies), ceruloplasmin, alpha-1 antitrypsin, and serum ferritin, and underwent ultrasound scanning of the abdomen, which was normal. This was followed by a percutaneous liver biopsy. Each biopsy was reviewed with particular attention to features of interface hepatitis, lobular necroinflammation and biliary changes. The patients were categorized according to the probability of having autoimmune hepatitis: no evidence (scores < 10), probable (scores of 10-15) or definite (score of more than 15), according to the established international criteria for diagnosis of autoimmune hepatitis[11,12].

All patients were treated with prednisolone (0.5 mg per kilogram per day) initially. Complete response, partial response and no response were defined according to the original and revised international autoimmune hepatitis criteria[11,12].

RESULTS

Acute hepatitis was defined in accordance with the scheme established by the Council for International Organisations of Medical Sciences (CIOMS)[13], and by the USFDA Drug Hepatotoxicities Steering Committee[14]. Eight patients were identified, of which six had presented with acute hepatitis on this basis. All were male and five of these patients were of Somali origin, while one patient was from Yemen. The age range of these patients was 24 to 57 years (mean 42.3 years). All of the patients had been using khat for several years. There was no history of herbal medication (other than khat) or alcohol use in any patient. All other causes of liver injury were excluded *via* non invasive liver screen. Five of the six patients went on to have a liver biopsy. The patients were scored according to the revised autoimmune hepatitis criteria - (Table 1). They were given -4 for khat use on the scoring system due to its potential hepatotoxicity. Despite this, five out of six patients had a pre treatment score of 10 to 15 which placed them in the probable group for autoimmune hepatitis.

The five patients that were in the probable group had at least a partial response to corticosteroids with a greater than 50% reduction in their ALT after one month of treatment. Only two patients had more than 1 year of follow up, with one showing complete response to treatment. The patient that had scored negative for AIH (< 10) showed the least improvement with prednisolone and continued to have raised liver enzymes after 1 year of treatment. Four out of the six patients were maintained on long term low dose prednisolone while the other two patients were lost to follow up after 1 year. Two patients were commenced on azathioprine with complete response at 1 year follow-up. There was no history of re-exposure to khat.

Five out of six patients met the criteria for probable diagnosis of AIH but none of the patients actually met the criteria for confirmed diagnosis (score > 15). It has been reported previously that Somalian patients with AIH present atypically. It is therefore suggested that the AIH in these patients may have been triggered by khat use.

DISCUSSION

Over 40 khat strains are grown and used in Southern Arabia and East Africa. It is consumed in the form of fresh leaves which may often be contaminated with pesticides. The leaves of khat contain the Pyrrolizidine alkaloids, Cathine, Cathidine, and Cathinone. The pleasure derived from khat chewing is attributed to the euphoric action of Cathinone which is a sympathomimetic amine, with properties similar to amphetamine[15]. Although Cathinone is restricted in the United Kingdom under the Misuse of Drugs Act 1971, khat possession and use are not[1].

The diagnosis of drug induced liver injury (DILI) *vs* AIH triggered by khat is challenging. Various causality methods have been used for herbal induced liver injury and can be broadly divided into retrospective and prospective methods[16-25]. Establishing with any degree of certainty as to whether the liver disease is drug-induced can be very difficult[26]. The issue is further compounded by the relatively rare incidence of DILI, under reporting and potential drug interactions, due to which establishing the identity of the culprit drug may be impossible[27,28]. Furthermore, histology is often unhelpful as it only provides the type and degree of liver injury rather than the

Table 1 Patient scores according to the revised criteria for diagnosing autoimmune hepatitis

Parameters	Score	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
ALP:ALT (or AST) ratio		125/1569	170/1223	187/1957	179/1005	298/1052	59/118
< 1.5	2	2	2	2	2	2	2
1.5-3.0	0						
> 3.0	-2						
Serum IgG above normal							
> 2.0	3	3		3	3	3	
1.5-2.0	2						2
1.0-1.5	1						
< 1.0	0		0				
ANA, SMA or LKM-1							
> 1:80	3			3		3	
Approximately 1:80	2				2		
Approximately 1:40	1						
< 1:40	0	0	0				0
AMA positive	-4	Negative	Negative	Negative	Negative	Negative	Negative
Viral hepatitis markers							
Positive	-3						
Negative	3	3	3	3	3	3	3
Drug History							
Positive	-4	-4	-4	-4	-4	-4	-4
Negative	1						
Average alcohol intake							
< 25 g/d	2	2	2	2	2	2	2
> 60 g/d	-2						
Liver histology							
Interface hepatitis	3	3	3	3	3	N/A	3
Lymphoplasmacytic infiltrate	1	1	1				1
Rosetting of liver cells	1	1	1	1			1
None of the above	-5						
Biliary changes	-3						
Other changes	-3						
Other autoimmune diseases	2	2 (IDDM)	0	0	0	0	0
Optional additional parameters							
Sero positivity-other antibodies	2					2 (ENA+)	
HLA DR3 or DR4	1	N/A	N/A	N/A	N/A	N/A	N/A
Pretreatment score		13	8	13	11	11	10

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; ANA: Antinuclear antibodies; SMA: Anti-smooth muscle antibody; LKM-1: Anti-liver/kidney microsomal type 1; AMA: Anti-mitochondrial antibodies; N/A: Not available.

aetiology. The key to causality is to assess the temporal relationship between drug initiation and development of abnormal liver tests and to diligently exclude other causes of liver diseases. This includes liver injury induced by alcohol, viral hepatitis (acute hepatitis A, B, C and E), autoimmune causes, metabolic disorders, biliary obstruction and sepsis.

The diagnosis of AIH alone is based on the characteristic clinical and histological features as well as the absence of other potential causes of hepatitis. The revised criteria for diagnosis of autoimmune hepatitis are considered the current gold standard[11,12]. Drugs can occasionally cause a clinical-serological picture similar to autoimmune hepatitis and may trigger autoimmune hepatitis in patients with an underlying genetic predisposition to autoimmune hepatitis, or the patients may develop AIH as a squeal of the drug itself. In a Swedish study of 23 patients who developed chronic DILI 23.1% were subsequently diagnosed with autoimmune hepatitis, the suspected drugs being ranitidine, enalapril, oestrogen, carbamazepine, and oestriol[29]. In a recent case series, Peevers *et al*[9] described seven patients presenting with acute hepatitis who had a history of khat use. Two of those patients met the criteria for the diagnosis of probable AIH. It has been reported previously that Somalian patients with AIH present atypically. In a study of Somalian patients with a history of AIH, it was noted that all of the patients were male and scored in the probable group[10]. History of chewing khat was not mentioned in that particular study. However, in our series, all of the patients who presented with acute hepatitis had a history of khat use with five out of six patient meeting the criteria for probable AIH and demonstrating a good clinical response to immunosuppression. We therefore conclude that, in addition to producing DILI, khat may also trigger AIH in patients with a possible genetic pre-disposition.

Recently, Terschke *et al*[30] have validated the use of the CIOMS scale to be used with herbal induced liver injury (HILI) cases. Although the diagnosis of AIH is well founded in these patients, the causality assessment by means of CIOMS is not available. Also, the small number of patients in this series means that our hypoth-

esis of AIH being induced by khat can only be tentative and should be interpreted with caution. Whether these patients benefit from long-term immunosuppression after stopping khat remains unclear. Further studies of similar groups of patients are required to increase our understanding of this phenomenon and its management.

COMMENTS

Background

Khat is widely used in Southern arabia and East Africa. It is also known that autoimmune hepatitis presents atypically in these population as it is more common in males and presents at a younger age.

Research frontiers

Khat is well known to cause liver damage but the mechanism of this remains elusive. Patients in the areas where khat is consumed, present with atypical autoimmune hepatitis - the cause of which is not known.

Innovations and breakthroughs

The authors present an interesting observation of development of autoimmune hepatitis in a group of patients consuming Khat.

Applications

People can treat these group of patients more effectively by understanding the possible mechanisms of liver damage caused by Khat use.

Terminology

Khat (Catha Edulis Celestrasae) is an evergreen shrub native to East Africa and Southern Arabia. It is chewed daily by over 20 million people in these countries for its addictive and euphoric properties.

Peer review

This is an important case-report and the manuscript reads well.

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CASE REPORT

Nested stromal-epithelial tumour of the liver: An unusual liver entity

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Core tip: Rare cases of hepatic nested stromal-epithelial tumours (NSETs), consisting of non-hepatocytic mixed stromal and epithelial neoplasms with associated calcification and ossification, have been previously described. To date, NSETs' behaviour and prognosis are completely unclear. We report the case of a 23-year-old female who underwent liver resection for a large hepatic, calcifying NSET. Details about preoperative imaging and the clinical and histopathological features of this very rare hepatic tumour are reported.

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Abstract

Nested stromal-epithelial tumours (NSETs) of the liver have been reported to be extremely unusual primary hepatic neoplasms. To date, few cases have been described in the literature. NSETs have been defined as non-hepatocytic and non-biliary tumours of the liver consisting of nests of epithelial and spindled cells, myofibroblastic stroma and variable intralesional calcification and ossification. Here, we report a case of a young female who underwent liver resection for a large hepatic lesion that proved to be a calcifying NSET on pathological examination. Details about the clinical and histopathological features of the tumour are reported.

Key words: Nested stromal-epithelial tumour; Hepatic tumour; Liver resection; Hepatic mixed tumour; Hepatectomy

INTRODUCTION

Nested stromal-epithelial tumours (NSETs) of the liver are very rare primary tumours characterised by unexampled clinicopathological features. To date, only isolated cases[1] and small series have been reported in the literature[2-4]. NSETs have been defined as a non-hepatocytic and non-biliary tumour of the liver consisting of nests of epithelial and spindled cells with associated myofibroblastic stroma and variable intralesional calcification and ossification[2]. This rare liver malignancy appears solid on imaging and has been reported to be isolated or occasionally associated with hormone cortisol-related syndrome. Herein, we describe a patient who underwent radical hepatectomy for a large calcifying NSET of the liver.

CASE REPORT

Clinical history

In January 2012, a 23-year-old female was referred to our

unit for recurrent dull abdominal pain associated with abdominal distension and dyspepsia. The patient had a past history of being negative for hepatitis but positive for the consumption of oral contraceptives during the previous 5 years.

On physical examination, a palpable mass in the upper abdomen was revealed. No Cushingoid or other clinical features were evident.

Laboratory tests revealed an altered serum level of aspartate aminotransferase (36 IU/L; normal range: 5-30), alanine aminotransferase (297 IU/L; normal range: 5-35), alkaline phosphatase (62 IU/L; normal range: 4-150) and gamma-glutamyltransferase (285 IU/L; normal range: 6-32). Virological markers for hepatitis B and C yielded negative results. Tumour markers, including carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP), were negative, whereas the level of carbohydrate antigen 19-9 (CA19-9) was elevated (98 IU/mL; normal value: < 40).

Abdominal computed tomography (CT) revealed a well-circumscribed liver lesion of 16 cm in size involving the left hemiliver, part of the right anterior section (S5-8)[5] and the paracaval portion of the caudate lobe (S1pc). Extensive vascular invasion including the left hepatic vein (LHV) and middle hepatic vein (MHV) at the hepatocaval confluence and the left portal pedicle (LPP) was evident. The lesion appeared solid and heterogeneous, with a rim-like enhancement on the arterial phase and a gradual centripetal enhancement on delayed phases. Multiple intralesional calcifications were also evident.

On magnetic resonance imaging (MRI), the tumour showed hypointensity on T1-weighted images and hyperintensity on both T2- and diffusion-weighted images (DWI), with small central necrotic collections. An inhomogeneous pattern with subcentimetric calcifications showing mostly hypointensity on both T1- and T2-weighted images was depicted. On gadolinium-enhanced images, the lesion showed a heterogeneous enhancement pattern on the arterial phase and washout in the portal and parenchymal phases (Figure 1A). The hepatocyte-specific delayed phase (Primovist, Bayer-Schering, Berlin, Germany) showed a hypointense lesion on T1-weighted images, with well-defined margins and a hyperintense capsule.

The work-up was completed with total-body ^{11}C-choline positron emission tomography (PET), which showed only slight pathological uptake of the tracer into the liver (Figure 1B). Based on these preoperative findings, a percutaneous lesion biopsy was not considered, and the patient was candidated to liver resection with a presumptive diagnosis of fibrolamellar hepatocellular carcinoma (HCC) or hepatocholangiocarcinoma.

At laparotomy, peritoneal carcinomatosis was excluded. During liver exploration, a huge, hard liver tumour entirely occupying the left hemiliver, part of S5-8 and the S1pc was confirmed. On intraoperative ultrasonography (IOUS), no additional lesions were detected, and the tumour showed well-defined margins and a heterogeneous echogenicity, with several intralesional hyperechoic spots due to multiple calcifications. Extensive involvement of the LHV, MHV and LPP was confirmed. Once the intraoperative staging was completed, the patient underwent an extended left hepatectomy with left and middle hepatic vein resection for radical removal of the mass. The specimen weighed 2000 g. Intraoperative blood loss was 100 mL, and the patient did not receive a blood transfusion. The postoperative course was uneventful, and the patient was discharge on the 10th postoperative day. Currently, the patient is alive and disease free 21 mo after surgery.

Pathological features

Grossly, the tumour had well-defined margins, was arranged in yellow lobules with several granular and rasping foci and was 16 cm in size (Figure 2A). On histology, the tumour was composed of well-defined nests of epithelial cells separated by strands of stromal cells and focally admixed with calcifying material (Figure 2B). The epithelial component was represented by polygonal to spindle-shaped cells, devoid of overt cytological atypia; stained positive for CKpool, CK19 and CD56; and stained negative for Hep Par-1 (hepatocyte paraffin 1) and AFP. Scattered nuclei were also immunoreactive for WT1 (Figure 2C). The stromal component stained positive for vimentin and smooth muscle actin. The mitotic index was < 5 mitoses/10 HPFs, and 10%-15% of cells stained positive for Ki67.

Molecular genetic study

Several neoplastic nuclei were positive for the EWS-WT1 fusion transcript (Figure 2D) but negative for the SYT-SSX fusion transcript.

DISCUSSION

Stromal-epithelial tumours are extremely rare conditions of the liver, and very few cases have been previously described[1-4]. The unusual entity defined as an NSET typically displays an arrangement of cellular nests composed of spindled or epithelioid cells surrounded by desmoplastic stroma and associated with variable calcifications or ossifications. To our knowledge, few cases of NSETs have been reported[1-4,6]: of these, only one has been described in Asian descendants[6].

This primary liver lesion represents an unexampled clinicopathological entity with an unclear pathogenesis. However, based on immunohistochemical studies showing an intimate correlation of tumoural cell nests with bile ducts and the expression of specific antigens, as well as the shared expression of CD56 by both components, the origin of this tumour in a hepatic mesenchymal precursor cell with primitive differentiation along the bile duct lineage is strongly suspected[3,4]. The expression of WT1 is also in favour of this hypothesis, as this multifunctional protein is a requisite component of the mesenchymal-to-epithelial transformation during certain processes in organogenesis[7].

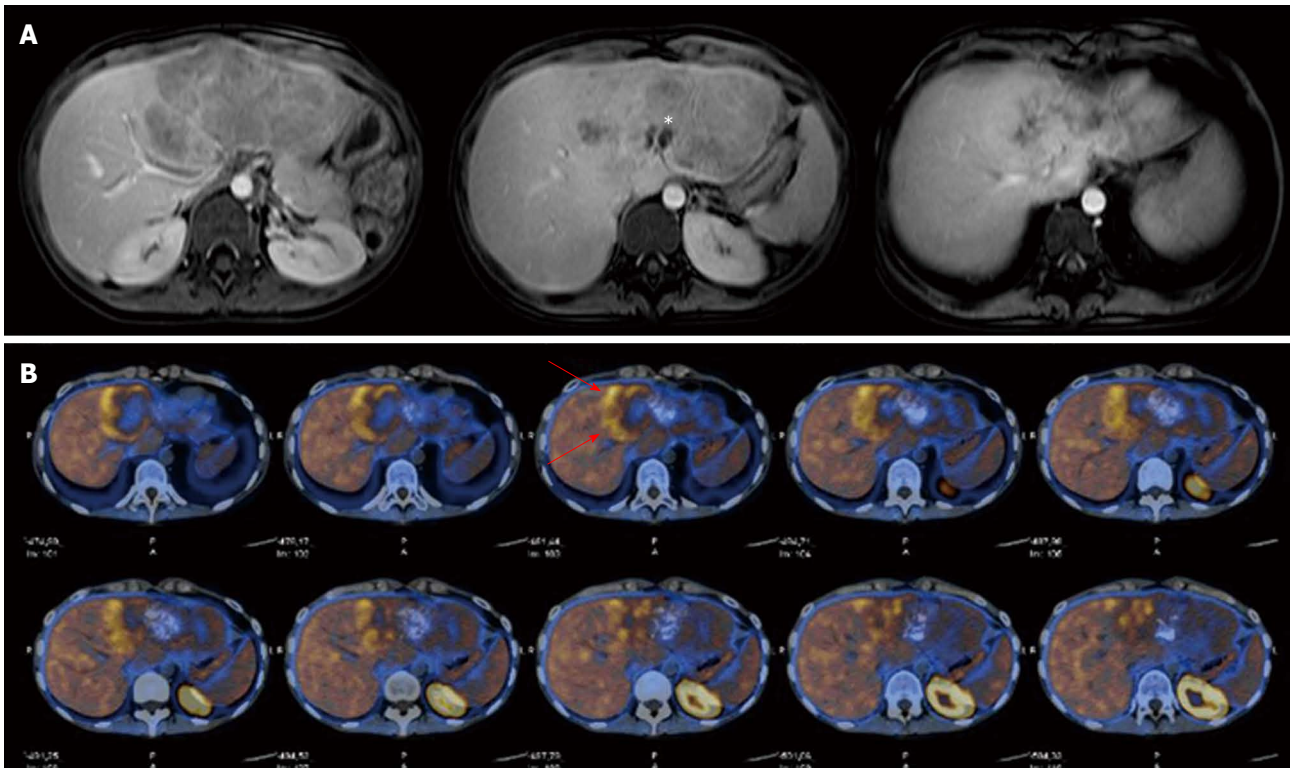

Figure 1 Radiological imaging. A: Abdominal magnetic resonance imaging scan showing a 16 cm diameter inhomogeneous and partially calcified (asterisk) mass of the left liver; B: Total-body ^{11}C-choline positron emission tomography showed a slight pathological uptake of the tracer in the peripheral part of the tumour (red arrows).

NSETs predominantly occur in young females and are more frequently located in the right hemiliver. Conversely, in the case described here, the tumour occupied the left hemiliver.

The development of this tumour may occur as an isolated condition or in association with a hormone-related syndrome, such as Cushing syndrome. Heerema-McKenney *et al*[3] reported two cases of NSETs occurring in paediatric patients that were associated with Cushing syndrome due to an elevated adrenocorticotropic hormone (ACTH) level[4]. Additionally, Rod *et al*[8] described a 17-year-old female patient affected by a large hepatic NSET causing mild Cushingoid syndrome secondary to moderate-to-high ACTH secretion. In our experience, Cushing-like symptoms were not evident.

From a clinical standpoint, one of the main differential diagnoses is a mixed epithelial and mesenchymal hepatoblastoma, although very few cases have been reported in adults[9]. However, this tumour shows components of foetal and/or embryonal hepatocyte differentiation and lacks the typical stromal architecture of NSETs. Synovial sarcomas and desmoplastic small round cell tumours (DSRCTs) are other diagnostic possibilities to consider that can be distinguished based on specific histologic features. Indeed, in the current study, the absence of a demonstrable carcinoma component and the SYT-SSX fusion transcript helped to exclude the diagnosis of synovial sarcoma. However, cases of synovial sarcoma with extensive calcification and osteoid formation have been reported[10,11]. In our case, the histologic features of the NSET were slightly reminiscent of those of DSRCTs[12,13], as both tumours exhibited nests of WT1-positive cells and were positive for the EWS-WT1 fusion transcript. The NSET, however, can be distinguished from DSRCTs by the NSET's typical arrangement of myofibroblastic collars and fibrovascular supporting stroma, which differed from the fibrous stroma of DSRCTs. However, the NSET was not immunoreactive for desmin, whereas the opposite is observed in DSRCTs. In the case reported here, based on preoperative imaging features, a diagnosis of fibrolamellar HCC or eventually mixed hepatocholangiocarcinoma was considered.

Interestingly, the patient had taken oral contraceptive pills (OCPs) during the previous 5 years. As reported for hepatic adenoma[14], a possible role for OCPs in the occurrence of the NSET could be considered. However, no staining for hepatocyte antigens was demonstrated in any tumour cells, which led us to exclude a likely correlation with hepatic adenoma. Furthermore, a lack of progesterone and oestrogen receptors in tumoural cells contributed to doubt about the hypothetical correlation between NSET occurrence and OCP consumption. However, more studies on a larger number of cases are likely needed before a possible correlation can be determined.

NSET prognosis remains an unclear matter, but based on current information, this tumour seems to have low proliferation activity and an indolent course, behaving as a low-grade malignancy featuring unusual extrahepatic spread and a possible presentation since childhood. However, specific tumoural features, a large size and vascular

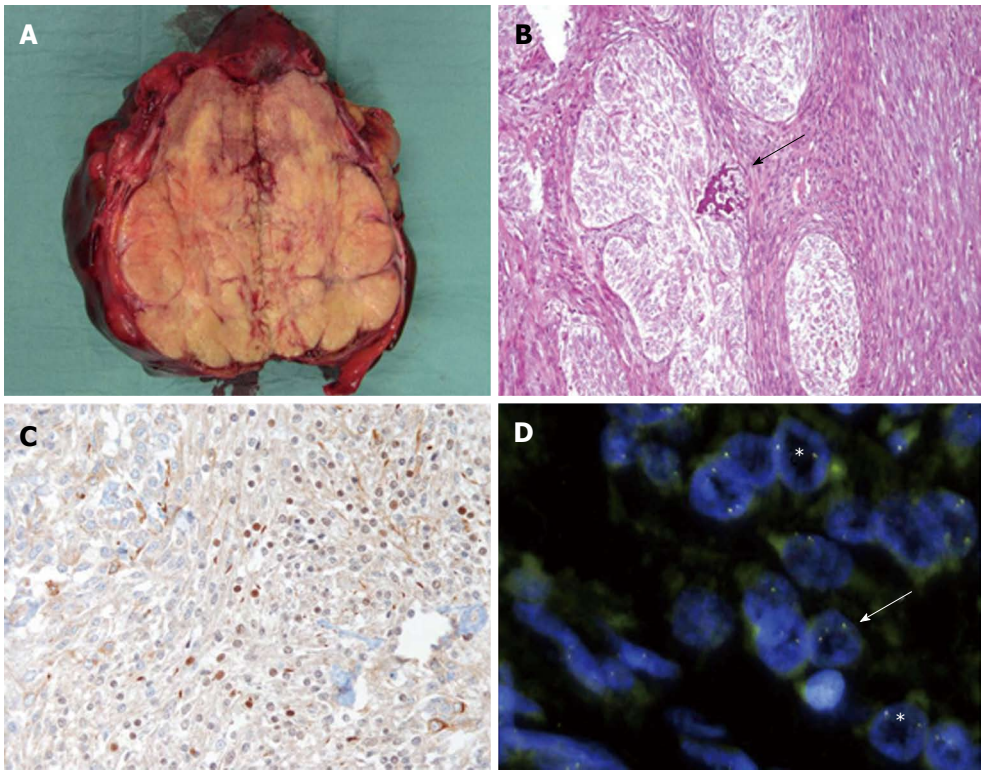

Figure 2 Pathological features of the reported calcifying nested stromal-epithelial tumor of the liver. A: At gross inspection the lesion shows well defined margins and is arranged in lobules; B: At histology the lesion is characterized by the presence of well defined nests of epithelial cells separated by strand of stromal cells, focally admixed with calcifying deposits (arrow); C: Scattered neoplastic cells stain positive for WT-1 immunostaining; D: Some neoplastic nuclei showed well separated green and orange probes signals (arrow), in keeping with a translocation involving *EWSR1* gene (FISH); some neoplastic nuclei (asterisks) did not show the same break apart pattern.

invasion seem to be associated with a higher probability of recurrence. Brodsky *et al*[15] reported experiences of recurrent NSETs of the liver with lymph node metastasis after partial hepatectomy. Hommann *et al*[16] reported a case of lung metastasis after liver transplantation for an unresectable NSET. Based on these experiences, although the risk of relapse seems negligible, a careful postoperative follow-up is recommended.

To date, the benefit of systemic chemotherapy and the most appropriate regimen to adopt remain poorly defined. In paediatric experience, preliminary results showing a minimal response for an unresectable NSET when a hepatoblastoma and sarcoma protocol regimen was adopted have been reported[3]. However, this topic remains completely unexplored.

Surgery seems to be the pivotal therapeutic approach, remaining the best strategy to guarantee longer survival and a better prognosis[1,3]. Our experience attempts to convey more information about the reliability of the surgical approach in the case of a resectable NSET. In this sense, our clinical experience confirms that liver resection allows the safe attainment of complete tumour clearance, even in advanced disease. Conversely, considering the low tendency of NSETs to relapse and previous unsuccessful experiences, at least for oncological control, liver transplantation generally should not be recommended, at least as a first choice[16]. Liver transplantation would be potentially useful for those patients with unresectable but not extrahepatic disease.

In conclusion, this report aimed to clarify the clinical history, therapy, imaging pattern and histopathological features of a very rare primary liver tumour that is still poorly characterised. Awareness of hepatic NSET occurrence may help to identify additional cases, enlarging knowledge about NSETs' clinical behaviour and prognostic features and limiting the possibility that these tumours could be misdiagnosed and confused with other aggressive liver malignancies.

COMMENTS

Case characteristics

Symptoms were featured by dull abdominal pain associated with abdominal distension and dyspepsia.

Clinical diagnosis

Palpable mass in the upper abdomen. Cushingoid clinical features can be sometimes detected.

Differential diagnosis

Mixed epithelial and mesenchymal hepatoblastoma, synovial sarcoma and desmoplastic small round cell tumour are the main differential diagnosis. Histologic and immunohistochemical analysis help to distinguish them.

Laboratory diagnosis

Laboratory tests revealed altered serum level of AST, ALT, alkaline phosphatase and gamma glutamyltransferase. Virological markers for hepatitis B and C and tumour markers including carcinoembryonic antigen, alpha-fetoprotein were negative, while, the CA19-9 was elevated.

Imaging diagnosis

Preoperative imaging (Computed tomography, Magnetic resonance imaging

scan) revealed a well-circumscribed liver lesion involving the left hemiliver. The lesion appeared solid and heterogeneous with a rim-like enhancement at contrast phase with multiple intra-lesional calcifications. An extensive vascular invasion was evident.

Pathological diagnosis

Nested stromal-epithelial tumours (NSETs) are a non-hepatocytic and non-biliary tumor of the liver consisting in nests of epithelial and spindled cells with associated myofibroblastic stroma and variable intralesional calcification and ossification.

Treatment

Surgery seems the pivotal therapeutic approach remaining the best strategy to guarantee longer survival and a better prognosis.

Related reports

Heerema-McKenney *et al*, Rod *et al* reported cases of NSETs occurring in pediatric patients and associated with a Cushing syndrome. Brodsky *et al* reported experiences of recurrent NSET of the liver with lymph-node metastasis after partial hepatectomy. Hommann *et al* reported a case of lung metastasis after liver transplantation for unresectable NSET.

Term explanation

WT1 is a mutifunctional zinc-finger protein involved in mesenchyme-to-epithelium transformation which suggests an origin of NSET in a hepatic mesenchymal precursor cell with primitive differentiation along the bile duct lineage. EWS-WT1 and SYT-SSX fusion transcript are genes can help to distinguish NSETs from other liver malignancy.

Experiences and lessons

NSETs occur predominantly in young female and their development may occur as an isolated condition, otherwise associated with hormone-related syndrome, such as Cushing syndrome. From a clinical standpoint, histologic and immunohistochemical studies are essential to distinguish NSETs from other malignancy because of lack of a typical imaging pattern.

Peer review

In the present study, the authors report a case with a nested stromal-epithelial tumor in the liver, which has been rarely reported worldwide. They fully examined the pathological features of the tumor, by using immunohistochemical analysis. This report has originality, figures are clear, and the discussion is well written.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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